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MOLECULAR ANALYSIS OF THE INTERACTION OF  
HERPES SIMPLEX VIRUS AND STEROID HORMONES  
IN EPITHELIAL CELLS

A thesis submitted for the degree of  
Doctor of Philosophy in the  
Faculty of Medicine

© Elizabeth Ann Offord (BSc)

Department of Biochemistry,  
University of Glasgow.

February, 1988.

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This thesis is dedicated  
to  
my parents



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## ABBREVIATIONS

The standard abbreviations recommended in the Biochemical Journal "Policy of the Journal and Instruction to Authors" (Biochem J 241: 11-21 (1987)) are used in this thesis with the following additions or exceptions:

|                   |   |
|-------------------|---|
| BFB               | bromophenol blue                                      |
| bp                | base pair(s)  |
| BSA               | bovine serum albumin                                  |
| BHK               | baby hamster kidney                                   |
| CAT               | chloramphenicol acetyl transferase                    |
| CIN               | cervical intraepithelial neoplasia                    |
| c.p.e.            | cytopathic effect                                     |
| c.p.m.            | counts per minute                                     |
| CS                | calf serum  |
| DCC               | dextran-coated charcoal                               |
| DBP               | DNA - binding protein                                 |
| DES               | diethylstilboestrol                                   |
| DMEM              | Dulbecco's modification of Eagle's medium             |
| DMS               | dimethylsulphate                                      |
| DMSO              | dimethylsulphoxide                                    |
| DNase             | deoxyribonuclease                                     |
| E                 | early   |
| E <sub>2</sub>    | oestradiol-17 $\beta$                                 |
| EGF               | epidermal growth factor                               |
| EIA               | enzyme immunoassay                                    |
| ER                | oestrogen receptor                                    |
| EtBr              | ethidium bromide                                      |
| <u>E. coli</u>    | <u>Escherichia coli</u>                               |
| FCS               | foetal calf serum                                     |
| GR                | glucocorticoid receptor                               |
| [ <sup>3</sup> H] | tritiated   |
| HIDCC-FCS         | heat-inactivated, charcoal-stripped foetal calf serum |

|                             |  |
|-----------------------------|--|
| HPV                         | human papilloma virus                                    |
| HS                          | human serum  |
| HSV                         | herpes simplex virus                                     |
| IE                          | immediate-early  |
| K                           | kilodalton(s)  |
| L                           | late   |
| mu                          | map units  |
| m.o.i.                      | multiplicity of infection                                |
| mol. wt.                    | molecular weight   |
| NP40                        | nonidet P-40   |
| N.P.T.                      | non-permissive temperature                               |
| OD                          | optical density  |
| ORG 2058                    | synthetic progestin                                      |
| p.a.                        | post-adsorption  |
| PBS                         | phosphate buffered saline                                |
| pfu                         | plaque forming unit(s)                                   |
| Pg                          | progesterone   |
| p.i.                        | post-infection   |
| PR                          | progesterone receptor                                    |
| RNase                       | ribonuclease   |
| *rpm                        | revolutions per minute                                   |
| SDS-PAGE                    | sodium dodecyl sulphate polyacrimide gel electrophoresis |
| TCA                         | trichloroacetic acid                                     |
| TIF                         | <u>trans</u> -inducing factor                            |
| tk                          | thymidine kinase   |
| TLC                         | thin layer chromatography                                |
| UV                          | ultraviolet  |
| <sup>v</sup> / <sub>v</sub> | volume per volume  |
| <sup>w</sup> / <sub>v</sub> | weight per volume  |

All other abbreviations (e.g. chemical names, buffer solutions) are explained in the text.

\*In this thesis, centrifuge speeds have been given in rpm. To convert to relative centrifugal force (RCF or 'g') values, the following formula should be used: (see next page)

$$RCF = 11.17(r) \left( \frac{\text{rpm}}{1000} \right)^2 \quad (r = \text{radius in cm})$$

The following conditions were used:

| <u>Speed</u><br>(rpm) | <u>Centrifuge</u>                              | <u>Rotor</u> | <u>Radius of</u><br><u>Rotor</u> (cm) |
|-----------------------|--|--------------|---------------------------------------|
| 1000 - 3000           | RT 6000B<br>Coolspin                           | H-1000B      | 18.67                                 |
| 5000 -<br>15,000      | Sorvall<br>RC-5B<br>Refrigerated<br>Centrifuge | SS 34        | 10.80                                 |
|                       |  | GSA          | 14.61                                 |
|                       |  | GS3          | 14.14                                 |
| > 30,000              | Sorvall<br>ORD 50<br>Ultra-<br>Centrifuge      | TV 865B      | 8.47                                  |

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## SUMMARY

Herpes simplex virus (HSV) is associated with the development of cervical cancer. Steroid hormones play a role in the normal development and in the pathology of the cervix. In view of the increased risk of developing cervical cancer in people taking the steroid - based contraceptive pill, possible interactions between steroid hormones and HSV were investigated.

The growth of HSV-2 in either primary ectocervical cells or in the breast cancer cell line, ZR-75-1, was shown to be independent of either oestradiol ( $E_2$ ) or progesterone (Pg), in the concentration range  $10^{-9}M$  to  $10^{-7}M$ .

The HSV IE activator sequence TAATGARAT (R = purine), which responds to the virion trans-inducing factor, Vmw 65, is partially homologous to the consensus binding site for PR found upstream of chicken egg-white genes. The ability of the hormones,  $E_2$  and Pg to modulate gene expression via the HSV TAATGARAT element was tested. MCF-7 or ZR-75 -1 cells were transfected with plasmids encoding the CAT gene under the control of an HSV IE promoter and upstream TAATGARAT elements, in the presence and absence of physiological concentrations of  $E_2$  or Pg. No significant differences in the level of CAT activity with or without hormones were seen.

Vmw 65 interacts with the TAATGARAT sequence in association with cellular factors. Specific complexes between nuclear protein(s) from MCF-7 and ZR-75-1 cells and TAATGARAT DNA were detected by gel retardation assays. Interestingly, the two cell lines tested showed different binding patterns, indicating differences in the cellular factors which recognize TAATGARAT.  $E_2$  treatment of cells did not result in any modification of the complexes formed with TAATGARAT. No virus induced protein, specific for TAATGARAT, was observed under the conditions used for these experiments.

Thus no effect of  $E_2$  or Pg on the HSV IE activator sequence, TAATGARAT, was seen in the gene expression and DNA-binding studies described here.

Biochemical and immunological steroid receptor assays were used to test the levels of ER and PR in MCF-7 and ZR-75-1 cells after E<sub>2</sub> treatment and/or HSV (-1 or -2) infection. E<sub>2</sub> treatment induced a 4- to 7- fold stimulation of detectable PR, indicating that the cells were responding as expected to E<sub>2</sub>. In contrast, both E<sub>2</sub> and HSV caused the level of ER to fall to about 10% of the value measured in control, untreated cells. In vivo, E<sub>2</sub> is known to stimulate the level of ER. A drop in detectable receptor may be due to modification of the receptor which prevents ligand binding or recognition by the monoclonal antibody. HSV also caused the level of PR to fall, indicating that E<sub>2</sub> and HSV have different mechanisms of action.

Attempts to in vitro translate and immunoprecipitate the ER protein from total cytoplasmic RNA were unsuccessful, despite the increase in ER mRNA levels detected in these samples in response to E<sub>2</sub> treatment or HSV infection (see later). The failure to detect ER by in vitro translation and immunoprecipitation was probably due to either the low abundance of the ER mRNA population or to the inability of the monoclonal antibody to recognize newly synthesized ER.

The possibility that HSV could influence steroid gene expression at the transcriptional level was tested by Northern blot and slot-blot analysis of ER mRNA levels. Changes in ER mRNA levels were correlated with the level of actin mRNA, taken as a standard message. E<sub>2</sub> stimulation ( $10^{-8}$ M E<sub>2</sub>, 24h) resulted in a 50% increase in ER mRNA levels. HSV-1 infection resulted in a time - dependent increase of ER mRNA, reaching up to 250% of the control level by 8h post-infection (10 pfu/cell). HSV-2 infection (10 pfu/cell, 6h) stimulated the ER mRNA level by only 30%. The lower stimulation by HSV-2 may reflect the fact that HSV-2 infection induced earlier detachment from the substrate than HSV-1. The combination of E<sub>2</sub> together with HSV gave a greater response than with either agent alone, showing that HSV and E<sub>2</sub> stimulate the level of the ER message by different mechanisms. Stimulation of the ER message may be by transcriptional activation or by message stabilization.

ER mRNA levels were 10 - 50% lower than the control value if cells were treated with cycloheximide for 30 minutes prior to and throughout the course of infection. This result suggests the involvement of both viral and cellular proteins in stimulation of the ER mRNA level.

The mutant virus, tsK, which synthesizes only IE polypeptides at the non-permissive temperature (including an abnormal form of the regulatory protein, Vmw 175), stimulated the level of ER mRNA by approximately 200 - 300%. Stimulation by tsK may be mediated by an IE protein(s) or by the trans-inducing factor, Vmw 65, present as a virion component. In some experiments, stimulation of ER mRNA was artifactually high (up to 600%) due to a significant depression of the actin message against which the ER message level was calculated. Depression of the actin message was not seen with wild-type HSV infection.

HSV or tsK infection is known to induce the heat shock response. The possibility that the increase in ER mRNA induced by virus infection was related to a general stress response was tested. The ER message was not induced by chemical stress. In one experiment, heat - shocked cells showed an increase in ER mRNA but this was not a repeatable result.

Three candidates for viral transcriptional activators are the IE proteins, Vmw 175 and Vmw 110 and the virion trans-inducing factor, Vmw 65. The ability of each of these proteins to stimulate the level of ER mRNA was tested in transfection experiments. No significant stimulation of ER mRNA by Vmw 175 or Vmw 110 was observed, even although these proteins activated the expression of an HSV E gene (tk) in a transient CAT assay system. Transfection of plasmids encoding Vmw 65 resulted in a 200 - 300% increase in ER mRNA levels, a similar level of activation as was found with HSV-1 and tsK infection. Transfection of plasmids encoding mutated forms of Vmw 65, which were unable to trans-activate the TAATGARAT element in a



transient CAT assay system, also resulted in a 200 - 300% increase in ER mRNA levels. Thus Vmw 65 functions differently in the method of activation of TAATGARAT sequences and the stimulation of ER mRNA levels.

The ability of HSV to stimulate the level of a cellular message of a protein directly involved in growth control may be important in view of the association of HSV with cervical cancer and the importance of transcriptional activation as a mechanism in oncogenesis. It is particularly interesting that the gene transcript shown here to be upregulated is the ER mRNA since increased sensitivity to E<sub>2</sub> is associated with a number of reproductive epithelial cancers.

## INTRODUCTION

The introduction has been divided into three major sections:

- A1. Mechanism of steroid hormone action.
- B1. Herpes simplex virus.
- C1. Role of steroid hormones and virus in the development and pathology of the uterine cervix.

A1.1      INTERACTION OF STEROID HORMONES AND INTRACELLULAR RECEPTORS

Steroid hormones circulate in the blood bound to plasma proteins. An equilibrium exists between the bound and free states. Free steroid enters the "target" cells by passive diffusion down a concentration gradient, but there is much current discussion on what really constitutes "biologically available" steroid (Moore et al., 1986). Target cells are defined by the presence of appropriate, specific, high affinity receptors which bind the hormone and mediate its biological effects. Each steroid hormone has its own high affinity receptor. Various models have been proposed for receptor mediated steroid hormone action.

A1.1.1      The Classical Model

The original "two - step" model of ER action proposed by Gorski et al. (1968) and Jensen et al. (1968) was based on the distribution of specifically bound [<sup>3</sup>H]-E<sub>2</sub> in the nuclear and soluble fractions of homogenized tissues. In this model, binding of E<sub>2</sub> to its "soluble" cytoplasmic receptor results in the formation of a hormone - receptor complex (step 1) which is then "translocated" to the nucleus (step 2).

Prior to translocation, the hormone - receptor complex undergoes a temperature - dependent activation step which results in increased affinity of the hormone - receptor complex for chromatin and DNA (Jensen et al., 1968, 1969). In the case of the ER complex, this involves the conversion of the 4S soluble receptor (8S in low ionic strength buffers) to the 5S form, which is the salt - extractable form recoverable from the nucleus (Jensen et al., 1969). The 5S form is a homodimer (Notides et al., 1981). In the cases of the GR (Vedeckis, 1983), the AR (Liao et al., 1973) and, in some species, the PR (Saffron et al., 1976; Chen and Leavitt, 1979), activation involves a decrease in sedimentation constant.

Following activation and translocation to the nucleus, the hormone - receptor complex is believed to interact with "acceptor" sites in the nucleus and modulate the expression of a specific set of genes.

#### Al.1.1.2      The Equilibrium Model

Since the proposal of the "two - step" model for oestrogen action, the subcellular distribution of "empty" and "filled" receptor has been questioned on the grounds that homogenization of tissue in large volumes of buffer can lead to artifactual localization of the receptor in the cytosol. Sheridan's group (Sheridan et al., 1979; Martin and Sheridan, 1980, 1982) has proposed that unbound receptor is in equilibrium, partitioned between the cytoplasm and nucleus according to the free water content of these intracellular compartments. Additionally, using the "thaw - mount" technique and autoradiography, Sheridan et al. (1979) showed that even at 0°C, extensive nuclear localization of specifically bound E<sub>2</sub> occurred within 5 minutes in intact rat uteri.

#### Al.1.1.3      The Nuclear Model

Several groups have reported purely nuclear localization of steroid receptors. Welshons et al. (1984, 1985) investigated the subcellular distribution of ER, PR and GR in rat pituitary tumour cells. Cytoplasm and nucleoplasm fractions were prepared by exposing the cells to cytochalasin B. Incorporation of radiolabelled ligands into each fraction was measured. Unoccupied receptors were found predominantly in the nucleoplasm fraction while the cytoplasm fraction contained only minimum levels of receptors. Incubation of whole cells in enucleation medium (Percoll, cytochalasin B and DMSO) did not significantly change the number of receptors per cell or the distribution of receptor between the cytosol and crude nuclear pellet in homogenized cells. Welshons et al. concluded that "empty" receptor is loosely attached to the nucleus and that tight nuclear binding occurs once hormone binds to the receptor.

King and Greene (1984) utilized a number of monoclonal antibodies (which recognize various epitopes of the ER) in an indirect immunoperoxidase technique on frozen, fixed sections of human breast tumours, human uterus, rabbit uterus and in MCF-7 cell cultures. Nuclear staining was observed in all cases. The results were reported to be uniform under a range of fixation conditions and temperatures, thus the nuclear staining was unlikely to be due to artifactual translocation of the receptor during fixation or processing of tissues or cells. Short - term treatment of MCF-7 cells or immature rabbits with oestradiol led to no increase in nuclear staining intensity. The conclusions of King and Greene were similar to those of Welshons et al. (1984, 1985).

As discussed by Raam (1986), the anti - ER monoclonal antibodies are epitope specific and may cross - react with other nuclear antigens. Moreover, the antibodies may not be able to distinguish between the multiple physiological forms of the receptor (untransformed, transformed and "processed" receptor). The antibodies may preferentially bind the activated form of the receptor.

Immunofluorescence studies on frozen sections of human breast cancer biopsies, using polyclonal antibodies against the ER (Raam et al., 1982; Tamura et al., 1984) showed predominantly cytoplasmic fluorescence although nuclear fluorescence was also observed. Translocation of the receptor from the cytoplasm to the nucleus was observed after treatment with E<sub>2</sub> (Raam et al., 1983).

Immunohistochemical and immunocytochemical studies performed with monoclonal antibodies against the PR (Gasc et al., 1984; Perrot-Applanat, 1985) indicated nuclear localization of the PR. Similar studies on the GR did not show a purely nuclear localization (Govindan et al., 1980; Papamichail et al., 1980; Bernard and Joh, 1984; Fuxe et al., 1985; reviewed by Gustafsson et al., 1987).

Studies on the subcellular distribution of the rat GR after transfection of cloned cDNA sequences into cultured cells (Picard and

Yamamoto, 1987) showed cytoplasmic localization of the untransformed receptor in the absence of serum and phenol red, which is a weak oestrogen (Berthois et al., 1986). Artifactual nuclear localization of receptor was observed in the presence of serum and phenol red. When 180 amino acids were removed from the hormone - binding carboxyl terminus, the receptor was localized in the cytoplasm, even in the presence of hormone. Two nuclear localization signals, NL1 and NL2, were identified in the amino terminus of the receptor. The NL1 sequence is perfectly conserved in the human (Weinberger et al., 1985) and the mouse (Danielson et al., 1986) GR's. Neither NL1 nor NL2 bound DNA in vitro (Rusconi and Yamamoto, 1987). Thus, DNA binding activity and nuclear localization of the receptor were separable functions (Picard and Yamamoto, 1987).

Differences in intracellular localization of steroid receptors may exist but it is important to check that the methodology used to examine the distribution of receptors does not give artifactual results.

## A1.2 PHOSPHORYLATION AND DEPHOSPHORYLATION OF STEROID RECEPTORS

### A1.2.1 Phosphorylation of Steroid Receptors

There is some evidence that certain functions of steroid receptors are regulated by phosphorylation and dephosphorylation of the receptor. The GR is phosphorylated in glucocorticoid responsive L-cells but not in glucocorticoid resistant L-cells (Housley and Pratt, 1983). The chicken oviduct PR can be phosphorylated in vivo (Dougherty and Toft, 1982) and the rabbit uterine PR undergoes a hormone - dependent phosphorylation in generation of the "nuclear" DNA - binding form of the receptor (Logeat et al., 1985).

Hormone binding of purified ER from calf uterus is inactivated by a nuclear phosphatase (Aurrichio et al., 1981; Migliaccio et al.,

1982) which removes phosphate from the receptor (Aurrichio et al. 1984). Hormone binding is reactivated by a calmodulin stimulated kinase that phosphorylates the receptor exclusively on tyrosine (Migliaccio et al., 1982, 1984). Purified ER from whole rat uterus labelled in vivo with [ $^{32}$ P]-orthophosphate is labelled on tyrosine (Migliaccio et al., 1986). The ER tyrosine kinase is regulated by physiological concentrations of  $E_2$  (Aurrichio et al., 1987).  $E_2$  increases the affinity of the kinase for its substrate, dephosphorylated receptor, and stimulates hormone binding to its receptor. The anti - oestrogen, tamoxifen, inhibits tyrosine phosphorylation and binding of  $E_2$  to its receptor (Aurrichio et al., 1987).

The complete cDNA sequence of the ER contains information for four tyrosyl and two seryl residues that are potential sites for phosphorylation (Green et al., 1986). Aurrichio et al. (1987) have suggested that protein tyrosine phosphorylation may be an initial event of oestrogen action on target tissues. Retroviral transforming proteins (Bishop, 1985), peptide hormones and growth factors are phosphorylated on tyrosine residues.

#### Al.2.2 Dephosphorylation of Steroid Receptors

Whereas phosphorylation is thought to play a role in hormone binding to the receptor, dephosphorylation may be involved in activation of the receptor to the DNA - binding form. Evidence for this comes mainly from studies with phosphatase inhibitors. Molybdate, a potent inhibitor of phosphatase, inhibits thermal activation of the GR (Leach et al., 1979; Barnett et al., 1980), the PR (Nishigori and Toft, 1980) and the ER (Shyamala and Leonard, 1980). These results are consistent with the idea that activation of the receptor involves dephosphorylation. However, several other phosphatase inhibitors (e.g. fluoride, arsenate and phosphate) do not inhibit activation of GR or PR (Barnett et al., 1980; Nishigori and Toft, 1980). Further, there are several possible ways in which molybdate may inhibit activation (Barnett et al., 1980; Nishigori and Toft, 1980)

including modification of protein activity through interaction with amino acid residues (e.g. thiol group of cysteine) or complexing of phosphate groups which may reside on the receptor or on a component associated with receptor activity. Molybdate may also inhibit serine proteases, a role for which has been suggested in activation of the GR (Hubbard et al., 1984) and ER (Puca et al., 1986). Inhibitor studies by Puca et al. (1986) have shown that a serine protease within the receptor molecule itself is stimulated by hormone binding. They proposed that this serine protease activity is responsible for activation of the receptor to the DNA - binding form.

### Al.3        ASSOCIATION OF HEAT SHOCK PROTEIN 90 WITH STEROID RECEPTORS

Two laboratories have reported that molybdate stabilized avian PR's are eluted from steroid affinity columns in association with a phosphoprotein of mol. wt. 90 000 that does not bind progesterone (Dougherty and Toft, 1984; Renoir et al., 1984). Monoclonal antibodies raised against the 90K phosphoprotein also interact with molybdate stabilized receptors for oestrogen, androgen and glucocorticoid hormones (Joab et al., 1984; Housley et al., 1985; Sullivan et al., 1985). Immunoblotting and peptide mapping studies (Catelli et al., 1985; Sanchez et al., 1985; Schuh et al., 1985) have shown that the 90K phosphoprotein is a heat shock protein (hsp 90) similar to that previously shown to be induced by heat or stress (reviewed by Welch et al., 1982).

The biological significance of the association of hsp 90 with steroid receptors is uncertain. Activation or transformation of the receptor is accompanied by dissociation of hsp 90 (Mendel et al., 1986). Thus hsp 90 only associates with the untransformed receptor. Based on the analogy of the retrovirus protein kinase association with hsp 90 (Brugge et al., 1981, 1983; Courtneidge and Bishop, 1982; Lipsich et al., 1982), a number of possible functions for hsp 90 in association with steroid receptors have been proposed (Catelli



et al., 1985; Schuh et al., 1985; Ziemiecki, 1986). A modulatory role for hsp 90 would be in keeping the steroid receptor in an inactive state either by blocking the DNA - binding domain or by altering its phosphorylation state. Intracellular transport of steroid receptors may be aided by hsp 90. The untransformed receptor may be stabilized by association with hsp 90.

Several factors argue against a specific function for hsp 90. The hsp 90 is an abundant cytoplasmic protein making up 1% of total cell protein which is about 100 times in excess of what is needed to account for binding to steroid receptors (Gasc et al., 1984). Moreover, hsp 90 is found in a wide variety of tissues and species (Riehl et al., 1985), not all of which are targets of hormone action. In addition, if the true location of hsp 90, in the intact cell, is in the cytoplasm and the true location of steroid receptors is nuclear, then the association of the two proteins in broken cell preparations may be coincident and simply reflect the "sticky" nature of one and the abundance of the other.

#### A1.4      MODULATION OF CELLULAR ER LEVELS

The modulation of cellular ER levels by steroid has been studied in the immature rat uterus and in MCF-7 cells (reviewed by Kassis and Gorski, 1983). A single injection of E<sub>2</sub> in the rat leads to a dose dependent loss of "soluble" receptor and a simultaneous rise in tightly bound nuclear receptor. The soluble receptors are then gradually "replenished" to control levels over a period of 11 - 16 h (Sarff and Gorski, 1971). By 32 h post-injection the receptor levels reach 150% of the control level, 70% of which is newly synthesised receptor and 80% of which is recycled receptor. Responsiveness of target tissues to further hormone treatment seems to be dependent on receptor replenishment (Anderson et al., 1974).

Replenishment of cytoplasmic receptor, after translocation to the

nucleus, lags behind nuclear receptor loss and thus the total, detectable receptor content is decreased 2 - 6 h post-injection (Kassis and Gorski, 1983). This apparent loss of receptor has been termed "processing" and occurs both in vivo and in vitro. This phenomenon has been extensively studied in MCF-7 cells.

Treatment of MCF-7 cells with E<sub>2</sub> results in translocation of the ER complex to the nucleus followed by a rapid loss of detectable nuclear receptor (70% loss within 3 - 5 h), not explained by a concomitant rise in cytoplasmic receptor (Horwitz and McGuire, 1978c, 1980). This loss or "processing" of receptor is inhibited by the specific G-C base pair intercalators, actinomycin D and chromomycin A<sub>3</sub> but not by inhibitors of RNA, DNA or protein synthesis although maintenance of low, steady-state levels of receptor does require protein synthesis (Horwitz and McGuire, 1978b, 1980). This suggests that interaction of the receptor complex with chromatin is important for processing to occur.

There is some evidence to suggest that processing may be an important step for oestrogen action. Antioestrogens allow accumulation of nuclear receptor complexes but inhibit processing (Horwitz and McGuire, 1978c). In addition, an oestrogen unresponsive variant of MCF-7 cells fails to show processing (Nawata et al., 1981). Induction of PR accompanies loss of ER (Horwitz and McGuire, 1978a). However, further investigation into the induction of PR showed no direct correlation with the extent of ER loss (Strobl et al., 1984).

Processing in MCF-7 cells involves both physical and kinetic modifications to the receptor. Continued exposure of MCF-7 cells to E<sub>2</sub> for 6h results in a tightly bound, less exchangeable form of the receptor with distinct molecular alterations (Kasid et al., 1984). Kinetic studies show two populations of receptor (Strobl et al., 1984). Generation of a rapidly dissociating population of oestradiol binding sites is followed by a loss of oestradiol binding capacity from these sites (reduced by 45% after 5h of incubation with E<sub>2</sub>).

In the presence of actinomycin D, only a single population of slowly dissociating receptor is detected. Thus processing in MCF-7 cells seems to result from the loss of rapidly dissociating, high affinity binding sites for oestradiol.

An equilibrium model for oestrogen replenishment has been proposed by Kassiss and Gorski (1983). The model describes three forms of the receptor:

- (a) an active form with high affinity for steroid
- (b) an inactive form with low affinity for steroid
- (c) a transformed state with high affinity for polyanions, chromatin proteins and DNA.

#### A1.5      CHANGES IN MACROMOLECULAR SYNTHESIS INDUCED BY OESTROGEN

##### A1.5.1    Rat Model

An injection of  $E_2$  into the rat results in a sharp rise in RNA polymerase II activity within 15 min followed by a rise in polymerase I activity 30 - 60 min after  $E_2$  injection (Glasser et al., 1972). Optimization of biosynthetic activity occurs through histamine release, eosinophil migration, increased nucleotide and amino acid uptake, glucose metabolism and vascular permeability (Clark and Peck, 1979). The stimulation of DNA synthesis is directly related to the rate of protein synthesis 12h after  $E_2$  injection, the retention of nuclear receptors and the amount of receptor processing taking place (Stack and Gorski, 1985). Sustained steroid induced growth requires that a minimum number of available acceptor sites in the nucleus remain occupied for at least 6 - 12 h. Saturation of all available sites is not necessary (Giannopoulos and Gorski, 1971; Anderson et al., 1973).

PR synthesis is induced by  $E_2$  in rat uterine tissue (Hsueh et al., 1975) and is dependent on RNA and protein synthesis (Leavitt et al.,

1979). Progesterone inhibits ER synthesis by interfering with replenishment of the unfilled receptor (Hsueh et al., 1976).

#### A1.5.2 Breast Cancer Cells

E<sub>2</sub> induces a large number of enzymes involved in nucleic acid synthesis in cultured breast cancer cell lines (e.g. MCF-7, ZR-75-1), (reviewed by Dickson and Lippman, 1987). Stimulation of MCF-7 cells with physiological concentrations of E<sub>2</sub> for 24h results in a 2- to 5-fold rise in tk activity (Bronzert et al., 1981). In methotrexate resistant MCF-7 cells, E<sub>2</sub> induces a 2-fold increase in dihydrofolate reductase (DHF) activity (Cowan et al., 1982). Regulation of tk and DHF activity by E<sub>2</sub> is at the mRNA level (Aitken et al., 1985; Kassid et al., 1986).

A number of mRNA's which are regulated by oestrogen have been identified by selective screening of cDNA libraries prepared from MCF-7 cells (Masiakowski et al., 1982; May and Westley, 1986) and from ZR-75-1 cells (Westley and May, 1987). Masiakowski et al. (1982) identified a cDNA clone, pS2, corresponding to a 600 - nucleotide mRNA whose synthesis is induced at the transcriptional level by the addition of E<sub>2</sub> to the culture medium. Induction of pS2 mRNA by physiological concentrations of E<sub>2</sub> in MCF-7 cells (2-fold induction) has been confirmed by others (Aitken et al., 1985; Kassid et al., 1986). May and Westley (1986) identified four oestrogen - regulated mRNA's, pNR-1, pNR-2, pNR-3 and pNR-4, in MCF-7 cells. Two of the messages, pNR-3 and pNR-4, were induced at least 8-fold by E<sub>2</sub>. It is probable that the pS2 and pNR-2 messages are the same as both pS2 and pNR-2 cDNA clones hybridize to RNA of the same size (May and Westley, 1986).

Cathepsin D mRNA is an oestrogen - regulated message (Westley and May, 1987). Cathepsin D is a lysosomal aspartyl endopeptidase whose biological function is thought to be catabolism of cellular proteins. The 46K (also known as 50K or 52K) oestrogen - regulated,

secreted protein (Westley and Rochefort, 1980) may be cathepsin D (Morisset et al., 1986).

PR synthesis is induced by E<sub>2</sub> in MCF-7 cells (Horwitz and McGuire, 1978a). The presence of PR as well as ER in breast tumours is considered to be the best marker of hormone - responsive disease (Leake et al., 1981, 1987). However, the mechanisms by which oestrogens induce PR synthesis and growth seem to be separate (Reiner and Katzenellenbogen, 1986) so that tumour cells may be able to induce PR while no longer being dependent on oestrogen for growth. The reverse may also be true since some breast tumours (about one-third) which are ER positive but PR negative respond to endocrine therapy (Leake et al., 1987).

E<sub>2</sub> induces the synthesis of some less well characterized proteins in breast cancer cells: . Plasminogen activator and collagenolytic enzymes (Butler et al., 1979; Huff and Lippman, 1984) are thought to contribute to tumour progression and growth by allowing the tumour to digest and traverse encapsulating basement membrane (Liotta et al., 1986). The cell surface receptor for laminin (Albini et al., 1986) is thought to mediate attachment of normal cells to basement membrane laminin (Liotta et al., 1986) and to contribute to cellular invasiveness of tumour cells in colonization of new host tissues. Several relatively abundant secreted proteins, whose functions are not clear, are also induced by E<sub>2</sub> (Burke et al., 1978; Ciocca et al., 1983; Westley and Rochefort, 1980).

#### A1.6      MECHANISMS FOR OESTROGEN - PROMOTED GROWTH

Administration of oestrogen increases DNA synthesis and cell proliferation in the rat uterus (Kaye et al., 1972; Stormshak et al., 1976). Oestrogen applied locally to the lumen of the rat uterus stimulates DNA synthesis but to a lesser extent than oestrogen administered systemically (Stack and Gorski, 1984). This suggests both direct and indirect mechanisms for oestrogen - promoted growth.

The effect of oestrogen on cell proliferation in established cell lines derived from oestrogen - responsive tumours (e.g. MCF-7 cells) is difficult to interpret (Sonnenschein and Soto, 1980). These cell lines require oestrogen stimulation to grow as tumours in vivo, yet in vitro, they often show a very poor response to oestrogen ( Shafie, 1980; Sonnenschein and Soto, 1980).

One possible reason for the poor response seen in vitro is the fact that phenol red, the pH indicator used in most cell media at concentrations ranging from 30 - 45  $\mu\text{M}$ , is a weak oestrogen with a binding affinity 0.001% that of  $\text{E}_2$  for its receptor and capable of inducing cell proliferation and induction of the PR (Berthois et al., 1986; Rajendran et al., 1987). Rajendran et al. (1987) have proposed that phenol red causes its oestrogenic effect through activation of unfilled receptors by interacting at a site distinct from the steroid binding site. These results may help to explain the variable degree to which MCF-7 cells are reported to be growth-stimulated by  $\text{E}_2$ .

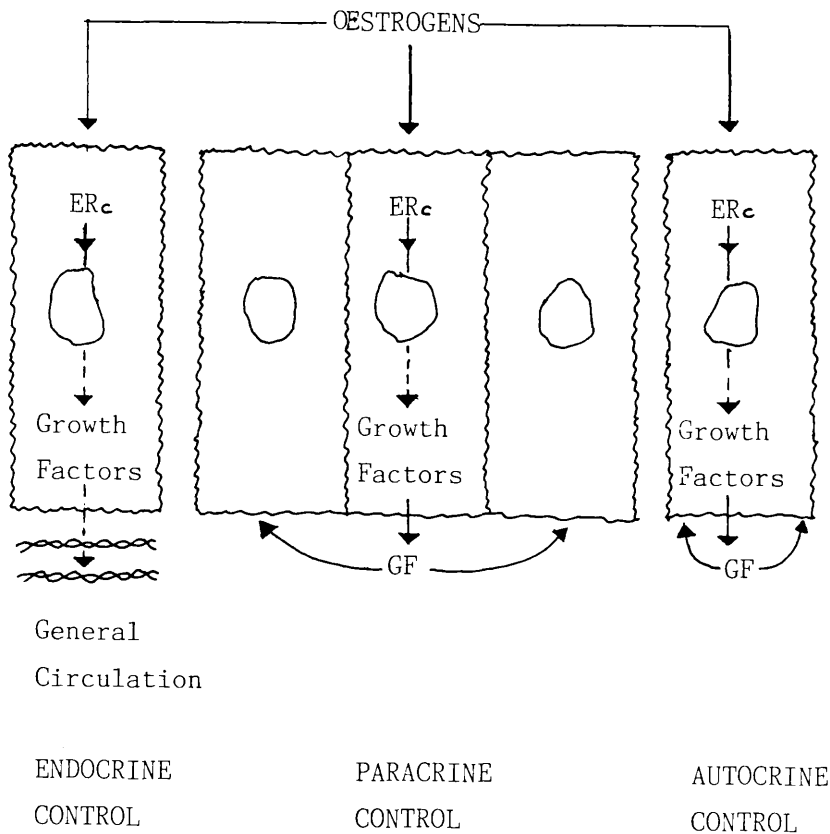
The different extent of growth response to topical and systemic oestrogen, observed by Stack and Gorski (1984), suggests that the "phenol red effect" does not fully explain the discrepancy between the level of response to  $\text{E}_2$  found in vivo and in vitro. This discrepancy has led to the proposal of indirect mechanisms for the role of oestrogen in inducing cell proliferation.

Sirbasku (1978) proposed that oestrogens trigger the synthesis and/or release of growth factors in the uterus, kidney or liver which would then be mitogenic for oestrogen - responsive cells. Extension of this model (Sirbasku, 1981; Ikeda et al., 1982) allows for three possible mechanisms (Fig. 1.1) :

- (a) A growth factor may be released from a stromal cell and act as a mitogen on a neighbouring epithelial cell (paracrine mechanism).

FIG. 1.1

PROPOSED MECHANISMS FOR INDIRECT MODELS  
OF OESTROGEN PROMOTED GROWTH



(Reproduced from Ikeda et al., 1982).

- (b) A growth factor may be released from the same cell for which it is mitogenic (autocrine mechanism).
- (c) A growth factor may be released into the general circulation and act on a distant cell.

Evidence for the secretion of growth factors from E<sub>2</sub> - stimulated breast cancer cells has been reviewed by Dickson and Lippman (1987). Briefly, oestrogen regulation of MCF-7 cells is associated with induction of the autostimulatory transforming growth factor  $\alpha$ (TGF- $\alpha$ ), induction of insulin-like growth factor 1 (IGF-1) and repression of the autoinhibitory transforming growth factor  $\beta$ (TGF- $\beta$ ). In addition, mammary stroma appears to provide an as yet unidentified factor(s) required for full mitogenicity of oestrogen (McGrath, 1983).

## A1.7 REGULATION OF GENE TRANSCRIPTION BY STEROID HORMONES

### A1.7.1 Role of Specific DNA Regulatory Sequences

#### A1.7.1.1 MMTV Model

Regulation of gene expression by glucocorticoids in the mouse mammary tumour virus (MMTV) has been studied as a model system for how activated hormone - receptor complexes interact with specific DNA regulatory sequences and possibly other nuclear factors to promote transcription.

Glucocorticoid hormones specifically increase the rate of synthesis of MMTV RNA in mouse mammary tumour cells and in MMTV - infected rat hepatoma cells while not appreciably affecting the overall rate of cellular RNA synthesis (Ringold et al., 1977). Hormonal regulation is maintained when cloned DNA fragments containing the MMTV LTR are transfected into cultured cells (Yamamoto et al., 1981; Groner et al., 1982). Regulation of transcription is at the level of initiation (Ucker et al., 1983; Firzlaff and Diggelmann, 1984).



The glucocorticoid responsive element (GRE) of the MMTV LTR can be distinguished and physically separated from the basal transcription initiation element (TIE), (Chandler et al., 1983). GRE activity persists even when its location and orientation relative to the TIE is changed. Transcription, in Ltk<sup>-</sup> cells, of plasmids containing increasing deletions at the 5'-side of the LTR linked to the coding region of the HSV tk gene has defined a fragment of about 200 nucleotides 5' to the transcription initiation site, as responsible for glucocorticoid responsiveness (Hynes et al., 1983; Buetti and Diggelmann, 1983; Majors and Varmus, 1983). Similar analysis of deletions progressing 3' to 5' has defined the border of the GRE to be between -59 and -65 of the LTR RNA initiation site (Ponta et al., 1985). A 184 nucleotide fragment of the LTR (-52 to -236) has purely regulatory properties (Ponta et al., 1985). This GRE confers inducibility to a tk gene and to an  $\alpha$ -globin gene, indicating that inducibility is not promoter specific. The GRE can function in a distance and orientation manner thus showing typical properties of an enhancer element (Chandler et al., 1983; Ponta et al., 1985)

Four GR binding sites (one distal and three proximal) have been identified between -200 and -70 in the MMTV LTR by DNase I footprinting (Scheidereit et al., 1983). These GR binding sites contain a common hexanucleotide sequence, 5'-TGTTCT-3', the guanosine residues of which are protected by the receptor protein from methylation by dimethylsulphate (Scheidereit and Beato, 1984), indicating that the TGTTCT sequence is involved in the contact between the hormone - receptor and the DNA double helix.

The hexanucleotide sequence is also present in the glucocorticoid responsive regions of the rat tyrosine aminotransferase (TAT) gene (Jantzen et al., 1987); the human metallothionein gene II (hMTII), (Karin et al., 1984); the 5'-flanking region, the first intron, the 3'-untranslated region of the human growth hormone gene (Moore et al., 1985; Slater et al., 1985) and the chicken lysozyme gene, inducible by

glucocorticoid, progesterone, oestrogen and androgen hormones (Renkawitz et al., 1984). However, there is some doubt about the functional significance of this sequence. The hexanucleotide sequence can be deleted from the hMT11 5'-flanking region without influencing the extent of hormonal induction (Karin et al., 1984) suggesting that, in this case, binding of GR to this sequence is not biologically relevant. In the case of the human growth hormone gene, only a region in the first intron shows the properties of a GRE and is the only region which gives a detectable exonuclease 111 footprint for GR binding (Slater et al., 1985). Thus the presence of the hexanucleotide sequence alone is not necessarily sufficient for glucocorticoid inducibility. In the case of the MMTV LTR, sequences flanking the TGTCT sequence are important for maximum glucocorticoid responsiveness (Kühnel et al., 1986).

#### Al.7.1.2 Recognition of the Same DNA Sequence by Different Steroid - Receptor Complexes

The chicken lysozyme gene contains two receptor binding sites for GR (Renkawitz et al., 1984). These sites are also recognized by PR (von der Ahe et al., 1985). The proximal binding site at -60 shows high affinity for GR and low affinity for PR, whereas the distal binding site at -180 has low affinity for GR and high affinity for PR. Deletions destroying the distal site result in parallel loss of inducibility by glucocorticoid and progesterone (von der Ahe et al., 1985). Exonuclease 111 protection experiments have shown that the region covered by GR is shorter than that covered by PR at the distal binding site (von der Ahe et al., 1986). DNase I footprinting and methylation experiments showed that the contact points between each receptor and the DNA double helix differ in 6 out of 7 positions (von der Ahe et al., 1986). Thus although GR and PR recognize the same regulatory sequences upstream of the chicken lysozyme gene, there are subtle differences in their recognition mechanisms. GR and PR also recognize the same sequences within the MMTV LTR (von der Ahe et al., 1985).

The MMTV LTR confers glucocorticoid and progesterone inducibility on homologous and heterologous promoters with each hormone acting through its own receptor (Cato et al., 1986). Androgen inducibility is also conferred by the MMTV LTR on adjacent genes provided the correct receptors are present in the cell (Darbré et al., 1986). The hormone responsive element (-236 to -52) within the MMTV LTR is sufficient to mediate progesterone and androgen induction of transcription (Cato et al., 1987) although progesterone stimulates transcription to a greater extent than androgen. The biological significance of induction of MMTV gene transcription by androgens is unknown. Progesterone is known to play a role in the progression of mouse mammary tumours.

#### Al.7.1.3    Role of A-T Rich Sequences

Examination of sequences upstream of the egg-white genes, expressed in chicken oviduct, for their ability to displace the PR complex from calf thymus DNA cellulose (Mulvihill et al., 1982) revealed the presence of strongly competitive fragments located 250 - 300 base pairs upstream of the start of transcription. The consensus sequence of these fragments, 5'-ATC<sup>CC</sup><sub>TT</sub>ATT<sup>A</sup><sub>T</sub>TCTG<sup>G</sup><sub>T</sub>TTGTA-3' was A-T rich. Preferential binding of PR to A-T rich sequences in the 5'-flanking region of egg-white genes was also found by Compton et al. (1983) using nitrocellulose filter binding assays with purified PR and specific labelled DNA fragments. The authors suggested that the receptor may act as a helix destabilizing protein within the A-T rich region. A similar model has been proposed by Sluyser (1983). Preferential binding of the ER complex to upstream regions of the chicken vitellogenin gene also occurs in an A-T rich region (Jost et al., 1984).

#### Al.7.2        Role of the Hormone in Receptor Recognition of Target DNA Sequences

Several groups (Bailly et al., 1986; Becker et al., 1986; Willmann and Beato, 1986) have investigated if the hormone has to be bound to

its receptor before the receptor can bind to its target DNA sequence and modulate transcription. Becker et al. (1986) carried out in vivo analysis of receptor - DNA interactions by the technique of genomic footprinting thus overcoming the problem of instability of the free receptor in vitro. They examined the interaction of GR with the upstream regulatory region of the rat TAT gene in TAT - expressing hepatoma cells and in rat fibroblast cells which do not express TAT. Dimethylsulphate reactivity patterns observed in vivo were compared with those obtained in vitro from methylated protein - free DNA in the presence and absence of dexamethasone. The in vivo analysis showed protection of guanosine residues in the binding site after treatment with dexamethasone in TAT positive but not in TAT negative cells, indicating that glucocorticoid increases the affinity of the receptor for its target sequence although this does not exclude the possibility that the unliganded receptor can interact with a GRE, albeit with lower affinity and selectivity. Many of the changes in dimethylsulphate reactivity observed in vivo were also found in vitro using cloned DNA and a partially purified GR. Local alterations in the structure of chromatin at the GRE's of the TAT gene take place as a consequence of hormone treatment as evidenced by the mapping of DNase I hypersensitive sites in this region (Jantzen et al., 1987).

Willman and Beato (1986) performed in vitro DNA binding experiments with crude receptor preparations from adrenalectomized rats. Cytosol was either hormone - free or precharged with a synthetic glucocorticoid or antagonist, activated by heating at 25°C for 30 min and the radiolabelled DNA probe added. Receptor - DNA complexes were footprinted or immunoprecipitated with mouse monoclonal antibodies against the receptor and the percentage DNA bound compared in control and steroid-treated cases. Specific binding of the receptor occurred in the presence and absence of hormone provided the receptor was heat - activated. Binding was inhibited by molybdate. The authors concluded that the hormone made no difference to the binding of receptor to its target DNA in vitro. They suggested that a possible function of the hormone may be to dissociate the unliganded receptor from any cellular

factors to which it may be bound (e.g. hsp 90 - discussed in section A1.3). It cannot be excluded that extensive purification of the receptor or in vitro heat activation may mimic the action of the hormone in causing a conformational change in the receptor, converting it to the DNA - binding form.

Bailly et al. (1986) studied the effects of ligand binding and receptor phosphorylation on the interaction of PR with specific DNA sequences in the rabbit uteroglobin gene using nitrocellulose filter binding and DNase<sup>I</sup> footprinting techniques. Binding sites were occupied with similar affinity by the receptor, either in its free state, or complexed with the hormone or an antagonist and by receptor that had been phosphorylated in vivo in a hormone dependent manner. In all cases, identical footprints were observed. The authors concluded that the hormone dependence of receptor binding to DNA or chromatin observed in intact cells and in crude cellular extracts cannot be seen with purified receptor. They made the same suggestion as Willmann and Beato (1986) that in vivo, the unliganded receptor may interact with some stabilizing factor which keeps it in the inactive form. The function of the hormone in vivo would be to break this interaction.

The results of Groyer et al. (1987) support the hypothesis that the role of the hormone in vivo is to permit transformation of the receptor to the DNA - binding form. The affinities of GR complexed with an agonist or an antagonist for DNA were compared. When the DNA - binding activity was measured relative to total receptor input, the affinity of the agonist - GR complex for DNA was greater than that of the antagonist - GR complex. However, when only the transformed (4S) form of GR was considered, the DNA - binding affinities of the agonist - GR and antagonist - GR complexes were identical. Thus it may be that the function of the hormone is to permit transformation of the receptor and that the antagonist acts by inhibiting this transformation rather than inhibiting binding of the hormone - receptor complex to DNA. Nuclear localization and DNA

binding activity are separable functions in the rat GR (Picard and Yamamoto, 1987; section A1.1.3).

### A1.7.3      Involvement of General Transcription Factors at the Promoters of Steroid Inducible Genes

Construction of a series of clustered point mutations and small deletions in the MMTV LTR regulatory region and quantitative analysis of their effects on hormonal stimulation of transcription (Buetti and Kühnel, 1986) showed regions important for maximum stimulation by glucocorticoid hormones. Mutations in the GR binding regions (section A1.7.1.1) had the greatest effect on hormone responsiveness. However, sequences around -70 and -30 (TATA homology required for efficient initiation of transcription) were required for maximum response but were not sufficient for hormone stimulation on their own.

The element at -70 includes all of the 5'-half of a sequence with dyad symmetry centred on position -70 that has been shown by Nowock et al. (1985) to bind a ubiquitous nuclear protein, termed "TGGCA - binding protein". A protein with similar properties, called "nuclear factor 1" (NF-1) enhances initiation of adenovirus DNA replication in vitro, but its function in vivo is not clear. The TGGCA consensus is also present in enhancers of certain papovaviruses (Nowock et al., 1985) and within a tissue specific enhancer identified upstream of the chicken lysozyme gene (Theisen et al., 1986).

Miksicek et al. (1987) tested the functional importance of the TGGCA sequence in a transient CAT assay system. The TGGCA sequence increased the level of hormone induced MMTV gene transcription but showed relatively stringent sequence requirements. The authors concluded that the TGGCA sequence constitutes an integral component of the MMTV core promoter and is required for efficient activity of the promoter and upstream regulatory region.

High resolution exonuclease III protection mapping of DNA - binding factors associated with the hormone - activated MMTV promoter in vivo

(Cordingley et al., 1987) confirmed the presence of a NF-1 binding site. In addition, a protein (F-i), probably involved in initiation of transcription, bound downstream of NF-1.

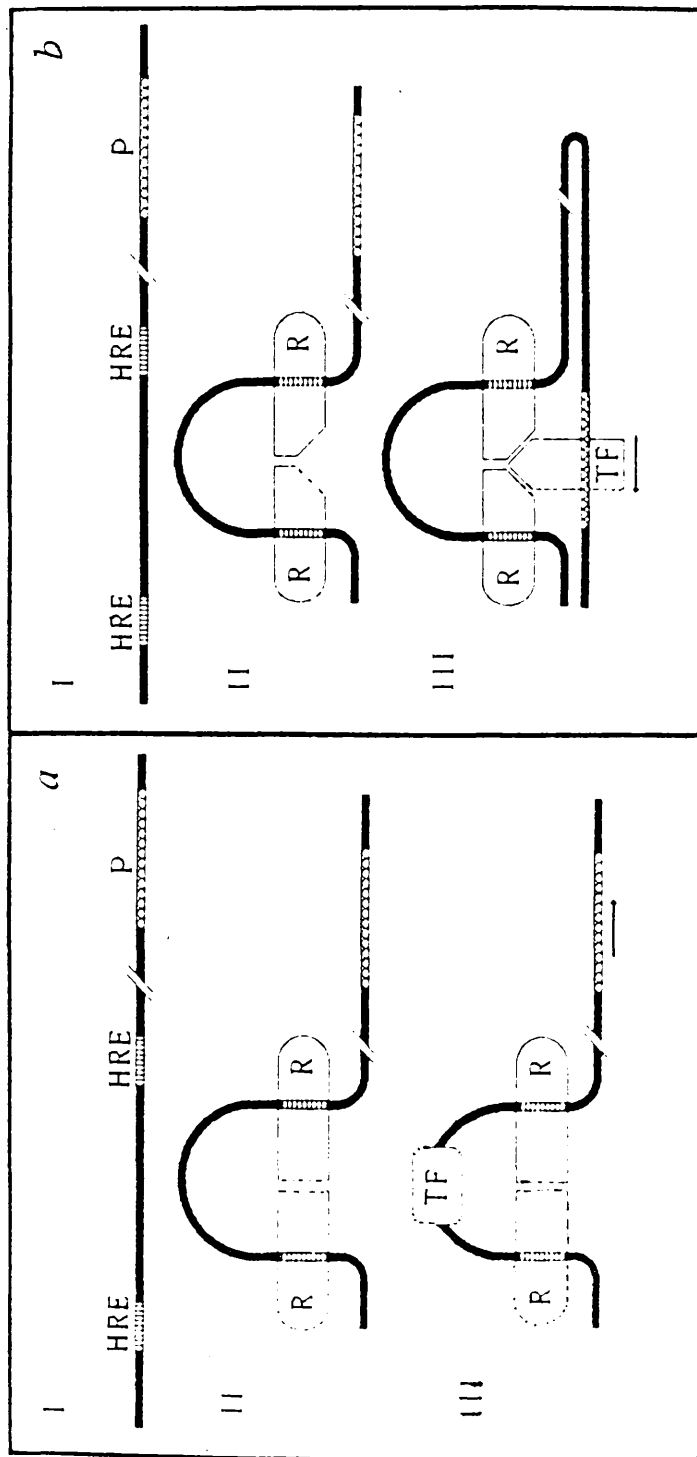
Possible models for the interaction of DNA - bound receptors and general transcription factors have been proposed by Théveny et al. (1987) based on electron microscopic studies of complexes formed between the PR and discrete regions of uteroglobin DNA (see fig. 1.2). Bound proteins may cooperate by transmitting a wave of conformational changes in the DNA or by establishing direct contact with each other, forming a complex which is recognized by the promoter.

#### Al.7.4      Role of Tissue Specific Transcription Factors

Expression of the egg-white proteins, conalbumin, ovalbumin and vitellogenin is differentially regulated in chicken oviduct and liver cells and is an excellent system for studying cell and tissue specific expression of genes. In chicken oviduct tubular gland cells, the vitellogenin 11 gene is not expressed while transcription of conalbumin and ovalbumin genes is regulated by oestrogen, progesterone, glucocorticoid and androgen hormones (Palmiter et al., 1972; Lee et al., 1978). In chicken parenchymal liver cells, transcription of the conalbumin gene is regulated by serum iron levels (McKnight et al., 1980a) and is much less sensitive to steroid hormones than in the oviduct (Lee et al., 1978; McKnight et al., 1980b). The ovalbumin gene is never expressed in these liver cells despite the presence of functional ER molecules which allow induction of vitellogenin 11 gene transcription (Deeley et al., 1977; Jost et al., 1978). Thus it is not just the presence or absence of functional steroid hormone receptors which determines how gene expression is controlled in specialised cells.

The ovalbumin promoter contains steroid independent elements located between -56 and -1 which require the presence of cell specific trans-acting factors to allow expression in embryonic hepatocytes

FIG. 1.2 POSSIBLE FUNCTION OF THE INTERACTION BETWEEN DNA - BOUND RECEPTORS  
IN THE REGULATION OF GENE TRANSCRIPTION





- (a) Model in which receptor oligomers (R) bind to both hormone regulatory elements (HRE) and associate to form a DNA loop. A transcription factor (TF) binds to the loop.
- (b) Model for receptor oligomers binding to both HRE's and forming a DNA loop. Contact between this receptor complex and TF enhances the binding of TF to the promoter region (P) or TF activity.

The direction of transcription is marked by an arrow.

(Reproduced from Theveny et al., 1987)

and oviduct tubular gland cells but not in adult hepatocytes or other cells (Dierich et al., 1987). A negative regulatory element(s) between -295 and -425 mediates the repression of the ovalbumin promoter region in oviduct tubular gland cells, possibly involving a specific "repressor" trans-acting protein (Gaub et al., 1987). Repression is relieved by steroid hormones.

The chicken lysozyme gene is also differentially expressed. In chicken oviduct tubular gland cells, the lysozyme gene is transcribed only when induced by steroid hormones, whereas in macrophages it is constitutively expressed and is not dependent on steroid hormones (Hauser et al., 1981; Sippel et al., 1986a). Theisen et al. (1986) have identified an enhancer element 6 kb upstream of the lysozyme gene which is functional only in cells expressing the lysozyme gene. The enhancer overlaps a DNase hypersensitive site which is present only in chromatin of cells naturally expressing the lysozyme gene (Sippel et al., 1986a, 1986b). These results suggest that interaction of a cellular factor(s) with the enhancer sequence changes chromatin from an inactive to an active state.

#### A1.7.5      Role of Non-histone Chromosomal Proteins

Spelsberg's group has provided evidence for the involvement of non-histone chromosomal proteins in the DNA/chromatin acceptor sites for steroid - receptor complexes (reviewed by Spelsberg et al., 1983). They have developed a cell - free nuclear binding assay in which chromatin is attached to an insoluble resin such as cellulose or acrylamide. Various protein fractions are removed, leaving residual DNA or DNA - protein complexes attached to the resins in an insoluble state. Hormone - receptor / chromatin binding studies can then be performed. Nuclear binding in the cell - free system is saturable and correlates well with that seen in vivo (Spelsberg et al., 1983).

Differential fractionation of chromatin proteins from avian oviduct and assay of PR binding to residual chromatin proteins has localized

PR binding activity to the 10% of non-histone proteins tightly bound to DNA which are removed by treatment with 7M guanidinium-HCl (Spelsberg et al., 1977, 1983). This fraction, termed CP-3 has been used in reconstitution experiments with DNA to form acceptor sites for PR and ER (Ruh and Spelsberg, 1983; Spelsberg et al., 1984). The reconstituted acceptor sites displayed saturable receptor dependent binding and patterns of binding (i.e. during the seasons and during oviduct development) similar to those found in vivo, whereas binding to pure DNA was non-saturable and did not reflect changes in the receptor binding pattern.

The acceptor activity of the CP-3 proteins was only seen when the bulk (80%) of the non-histone proteins (fraction CP-2) was removed with 4M guanidinium-HCl (Spelsberg et al., 1977, 1983). It seems therefore that the CP-2 proteins "mask" the CP-3 acceptor proteins thus preventing receptor complex interaction with DNA leading to regulation of gene expression. The CP-2 proteins have been further characterized and masking activity has been found in a fraction (CP-2b) in the molecular weight range 60 000 to 150 000, which is able to "remask" unmasked nucleoacidic protein (Dani and Spelsberg, 1985).

A possible physiological function of masking proteins is to allow differential gene expression by the same hormone and receptor during growth and development of the animal since the degree of masking of acceptor sites for the avian oviduct PR varies during cytodifferentiation of the oviduct (Spelsberg et al. (1983). It may also allow the same hormone and receptor to regulate different genes in different tissues in the same animal.

Chromatin fractions from rabbit uterus (Singh et al., 1984), calf uterus (Ruh et al., 1981; Ross and Ruh, 1984) and hen oviduct (Ruh and Spelsberg, 1983) all bind homologous ER complexes with high affinity. The tissue and species specificity of acceptor site non-histone proteins for ER binding has been tested by Ruh et al. (1986) by cross-reacting unmasked chromatin fractions and ER's

derived from several different shark or mammalian tissues. The only combination that revealed significant binding was shark testicular ER with shark testicular chromatin. Binding of shark testicular ER complex to rabbit chromatin was minimal. Similarly, ER complex from rabbit uterus, shark oviduct or mouse testis bound minimally to shark testicular chromatin. These studies suggest that unmasked chromatin acceptor sites are tissue and species specific.

#### Al.7.6      Conclusions on the Factors Important for Steroid Gene Expression

The factors involved in regulation of steroid gene expression are clearly complex. Specific DNA regulatory sequences and non-histone chromosomal proteins constitute the acceptor site for the activated hormone - receptor complex. Interaction of the hormone - receptor complex with DNA may be governed by cell and tissue specific factors. General and tissue specific transcription factors may cooperate with the activated hormone - receptor complex to induce maximum transcription. The role of the hormone appears to be in conversion of an inactive receptor to an active one capable of binding to chromatin/DNA.

## A1.8      DOMAIN STRUCTURE OF STEROID HORMONE RECEPTORS IN RELATION TO THEIR cDNA SEQUENCES

The human ER (Walter et al., 1985), chicken ER (Krust et al., 1986), rat ER (Koike et al., 1987), human GR (Hollenberg et al., 1985), rat GR (Miesfeld et al., 1984), mouse GR (Danielson et al., 1986), the human mineralocorticoid receptor (hMR), (Arriza et al., 1987), rabbit PR (Misrahi et al., 1987) and the human PR (Conneely et al., 1986, 1987; Jeltsch et al., 1986; Gronemeyer et al., 1987) cDNA's have all been cloned and sequenced. Comparison of the deduced amino acid sequences has revealed an overall domain structure for steroid receptors and homology to the avian erythroblastosis virus (AEV) p75 gag-erb-A fusion protein (Weinberger et al., 1985; Green et al., 1986), (see fig. 1.3). The v-erb A protein potentiates the action of v-erb B in transforming erythroblasts (Frykberg et al., 1983; Yamamoto et al., 1983). The cellular counterpart, c-erb A, has been shown to be a thyroid hormone receptor (Sap et al., 1986; Weinberger et al., 1986). The homology between steroid receptors and v-erb A suggests there may be a family of potentially oncogenic hormone receptors (Green and Chambon, 1986). The receptors for vitamin D<sub>3</sub> (McDonnell et al., 1987) and retinoic acid (Petkovich et al., 1987) also show the same domain structure as steroid receptors and homology to v-erb A.

### A1.8.1      ER cDNA

ER cDNA has been cloned from the human breast cancer cell line, MCF-7 (Walter et al., 1985) and shown to hybridize to a 6.2 kb mRNA. In vitro translation of ER mRNA in HeLa cells produces a protein of mol. wt. 65 000 with binding affinity for E<sub>2</sub> similar to that of the endogenous ER of MCF-7 cells (Green et al., 1986). The ER mRNA is 6322 nucleotides long with a 5'-untranslated region of 232 nucleotides and a 3'-untranslated region of 4305 nucleotides. The major open reading frame, which initiates at the second ATG, is 1785 nucleotides long and codes for a protein of 595 amino acids of mol. wt. 66 182 (Green et al. 1986). The chicken ER cDNA contains a major open reading frame coding for a protein of 589 amino acids of mol. wt. 66 669 (Krust et al., 1986). The mRNA is 7.5 kb and has a 3'-untranslated region.

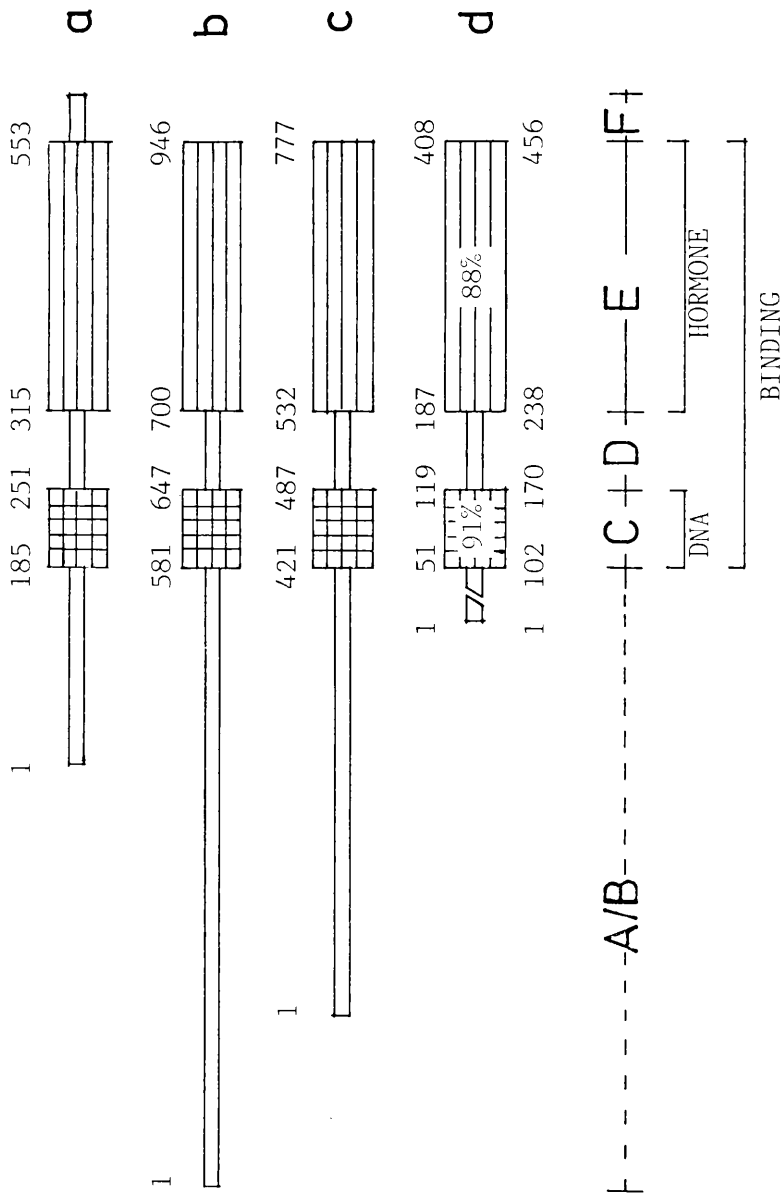


FIG. 1.3

DOMAIN STRUCTURE OF STEROID AND THYROID HORMONE  
RECEPTOR AMINO ACID SEQUENCES

Fig. 1.3 shows a schematic alignment of steroid and thyroid hormone receptor sequences (reproduced from Green and Chambon, 1986). The amino acid sequences, deduced from their complementary DNA sequences, of (a) human ER, (b) chicken PR, (c) human GR and (d) both the chicken (1 - 408) and human (1 - 456) c-erb A proteins are aligned on the basis of amino acid sequence similarity. The numbers refer to the positions of amino acid residues.

The division of the steroid hormone receptors into 6 regions (A - F), based on a comparison between the chicken and human ER's, is shown at the bottom of the figure. The two highly conserved regions, representing the putative DNA - binding (region C) and hormone - binding (region E) domains, are shown as shaded blocks. Little or no significant homology is observed when comparing other regions of the receptors (solid black lines).

Comparison between the chicken and human c-erb A protein sequences shows 91% and 88% sequence identity in regions C and E, respectively. Comparison between the various receptors in regions C and E show the following homologies:

|                              | <u>Region C</u> | <u>Region E</u> |
|------------------------------|-----------------|-----------------|
| steroid receptors/c-erb A    | 45 - 55 %       | 20 %            |
| human ER/chicken PR/human GR | 62 %            | 30 %            |
| chicken PR/human GR          | 91 %            | 55 %            |

The rat ER cDNA has a long open reading frame coding for a protein of 589 amino acids of mol. wt. 67 029. The mRNA is 6 kb and contains a 5'-untranslated region of 210 nucleotides and a 3'-untranslated region of 74 nucleotides (Koike et al., 1987).

Comparison of the human (h), chicken (c) and rat (r) amino acid sequences shows extensive homology (Krust et al., 1986; Koike et al., 1987). Based on the cER sequence, regions termed A, C and E show 87%, 100% and 94% homology respectively between the cER and hER (fig. 1.3). Overall sequence homology between rat and human ER is 88% and between rat and chicken ER is 77%. The N-terminal region of rER (amino acids 1 - 32) is 100% homologous to hER and 84% homologous to cER. Region C of hER and cER is 100% homologous to rER. Region E in the C-terminus of rER (amino acids 307 - 557) is 96% identical with hER and 94% with cER.

Such high conservation of sequence suggests the presence of important functional domains. Region A corresponds to the N-terminal end and may play a regulatory role in transcription. Region E is the C-terminal end and is strongly hydrophobic. Expression of deletion mutants of region E indicates that this region is indispensable for high affinity binding of E<sub>2</sub> and that the C-terminal half of the protein can bind hormone independently of the N-terminal half of the protein (Kumar et al., 1986).

Region C is hydrophilic and is rich in the basic amino acids cysteine lysine and arginine. This region is believed to be the DNA - binding domain (Green et al., <sup>1986</sup> Krust et al., 1986). Expression of deletion mutants in region C both in vivo and in vitro has shown that this region is important for tight nuclear association of the ER complex (Kumar et al., 1986). Moreover, this region contains potential DNA binding "fingers" in which four cysteines (or histidines) may be tetrahedrally coordinated with a Zn<sup>2+</sup> ion, allowing the intervening amino acids to form a finger which could make specific contact with the DNA (Miller et al., 1985; Berg et al., 1986). At least two highly conserved motifs of this type are contained in region C



of the ER, GR and PR amino acid sequences (Jeltsch et al., 1986; Krust et al., 1986).

Replacement of two cysteines by two histidines in the first potential DNA - binding finger of the hER prevents it from activating gene transcription (Green and Chambon, 1987). A chimaeric receptor formed by replacing region C of hER with that of the hGR activates expression of a glucocorticoid inducible gene but not of an oestrogen inducible gene, in the presence of E<sub>2</sub> (Green and Chambon, 1987).

As previously discussed (section A1.7.1.2), the GR and PR, which are 91% homologous within region C (Jeltsch et al., 1986), recognize common DNA - binding sites and activate a common hormone responsive element (von der Ahe, 1985, 1986; Cato et al., 1986). As the hER does not activate the glucocorticoid responsive MMTV-CAT reporter gene (Green and Chambon, 1987), it has been suggested that the conserved amino acids in region C provide the structural scaffolding of this domain, whereas the variable amino acids determine the specificity of the receptor - promoter interaction (Green and Chambon, 1987).

#### A1.8.2      GR cDNA

The complete amino acid sequence of the hGR has been deduced from cDNA clones (Hollenberg et al., 1985). Sequence analysis predicts two forms of the receptor,  $\alpha$  and  $\beta$ , probably generated by differential splicing. The sequences diverge after residue 727 with an extra 50 amino acids in the  $\alpha$ - sequence and 15 amino acids in the  $\beta$ - sequence. Both cDNA's contain a long 3'-untranslated region. Northern blot analysis of cytoplasmic mRNA from a human fibroblast cell line shows multiple mRNA's of 5.6, 6.1 and 7.1 kb, apparently due to alternative RNA splicing and polyadenylation.

In vitro translation of GR mRNA results in a protein of mol. wt. 94 000 which is identical in size to the native receptor, reacts with

receptor - specific antiserum and binds glucocorticoid with high affinity. The  $\beta$ - form of the receptor does not bind steroid (Hollenberg et al., 1985).

The domain structure of the receptor has been deduced from analysis of the primary amino acid sequence (Weinberger et al., 1985). The immunogenic domain which contains the epitope recognized by receptor - specific antiserum is found near the N-terminus (amino acids 145 - 278). The steroid - binding domain is in the extreme C-terminal end since  $\alpha$ -GR binds dexamethasone but  $\beta$ -GR does not. The region from amino acid 421 - 481 is rich in the basic amino acids, cysteine, lysine and arginine and shows 40% homology with the putative oncogene, v-erb A. This is considered to be the DNA - binding region.

Functional analysis of the domain structure of the hGR (Giguère et al., 1986; Hollenberg et al., 1987) by the use of deletion and linker insertion mutants, has confirmed that the C-terminus contains the steroid - binding domain and the region rich in basic amino acids is essential for transcriptional activity. In addition, two other regions affect transcriptional activity; one in the major immunogenic domain and one in the "hinge" region between the steroid- and DNA- binding domains (Giguère et al., 1986). These regions may interact with general transcription factors or may have an effect on the conformation of the activated receptor.

Detailed analysis of the limits of the DNA- and hormone- binding domains in the 795 amino acid rat GR (rGR), (Rusconi and Yamamoto, 1987) has shown that the terminal 30% of the receptor is important for hormone binding, in agreement with the results of Giguère et al. (1986). Sequence and functional analysis of the mouse GR (Danielson et al., 1986) has shown a domain structure similar to that of the rGR and hGR. Comparison of mouse and human GR's shows 89% homology overall (Danielson et al., 1986).

Deletion of the immunogenic or entire N-terminal domain of the hGR (Hollenberg et al., 1987) results in a constitutive receptor.

Deletion of increasing numbers of C-terminal and N-terminal amino acids from the rGR (Godowski et al., 1987; Miesfeld et al., 1987) while keeping the DNA - binding domain intact, results in a constitutively active receptor. These results imply that the DNA - binding domain is structurally autonomous and mediates constitutive enhancement of transcription in the absence of the steroid - binding domain. The results also suggest that the steroid - binding domain has an inhibitory effect on the DNA - binding domain.

Two general mechanisms by which the binding of hormone to its receptor results in the enhancer - activating protein have been proposed (Godowski et al., 1987):

- (a) Ligand - receptor interaction triggers a conformational change in the protein which results in the formation of the DNA - binding or enhancer - activation domain.
- (b) The ligand - stimulated structural transition might unmask (derepress) the DNA - binding or enhancer - activation domain already folded in the functional configuration.

The above results favour model (b).

### A1.8.3     PR cDNA

Partial cDNA sequences of the chicken (c) PR cDNA have been reported (Conneely et al., 1986; Jeltsch et al., 1986). The complete cDNA sequence of the cPR is now known and functional domains identified (Conneely et al., 1987; Gronemeyer et al., 1987). The rabbit (rb) PR has also been cloned and sequenced (Loosfelt et al., 1986). The human (h) PR amino acid sequence has been deduced from partial cDNA sequences but the full cDNA sequence has not yet been published.

Comparison of the cPR, hPR and rbPR amino acid sequences (Conneely et al., 1987; Gronemeyer et al., 1987) shows 100% conservation of sequence in the cysteine, lysine, arginine rich region C (by analogy with the ER domains, Krust et al., 1986), spanning amino acids 410 - 495 of the cPR. Region E from amino acid 540 to the C-terminus is

87% conserved. Region D shows 60% homology. The N-terminal region A/B of these PR's diverges significantly both in sequence and length.

Functional analysis of the domain structure of the cPR (Coneely et al., 1987; Gronemeyer et al., 1987) shows a similar domain structure for PR as for ER with region C being responsible for tight nuclear PR binding and region E being required to bind the hormone.

#### A1.8.4      v-erb A, c-erb A and Steroid Receptors

Avian erythroblastosis virus (AEV) belongs to the group of retroviruses capable of inducing rapid leukaemias and sarcomas in vivo. The genome consists of a cell - derived insert of approximately 3 kb, termed v-erb, flanked by remnants of the retroviral gag and env genes. Two proteins are generated by differential splicing of genomic RNA; a fusion protein of mol. wt. 75 000 (p75 gag-erb A) and a protein of mol. wt. 65 000 - 68 000 (v-erb B). The v-erb B protein is homologous to a portion of the epidermal growth factor receptor (Downward et al., 1984). The v-erb B protein is sufficient on its own to induce transformation of erythroblast cells while v-erb A potentiates the action of v-erb B by causing early blockage of cell differentiation within the erythroid lineage (Frykberg et al., 1983; Yamamoto et al., 1983).

The v-erb A gene has been sequenced (Debuire et al., 1984) and shows no comparison with the amino acid sequences of other known oncogenes. However, the N-terminal domain of v-erb A contains elevated levels of basic amino acids and is highly homologous to the putative DNA - binding domains of steroid receptors (reviewed by Green and Chambon, 1986; illustrated in fig. 1.3), suggesting the existence of a super-family of potentially oncogenic steroid receptor genes.

The cellular counterpart of v-erb A, termed c-erb A has been cloned from a chicken embryonic cDNA library (Sap et al., 1986) and from two placenta cDNA libraries (Weinberger et al., 1986). In both cases, the protein encoded by c-erb A was shown to be a high affinity receptor for thyroid hormone.

Recent studies by Glass et al.(1987) have provided evidence that the transcriptional effects of triiodothyronine ( $T_3$ ) can be mediated by the product of the c-erb A gene. A 16 bp sequence, located 164 bp upstream of the rat pituitary growth hormone gene, is sufficient to confer  $T_3$  responsiveness to the HSV tk promoter in transfected rat pituitary cells and specifically binds an in vitro translation product of the human placental c-erb A gene (Glass et al., 1987).

B1.1CLASSIFICATION

The family Herpesviridae has been divided into three subfamilies (Alpha-, Beta- and Gamma-) on the basis of biological properties such as host range, length of the reproductive cycle, cytopathic effect (cpe) and the ability to go latent in certain tissues (Matthews, 1982; Roizman, 1982).

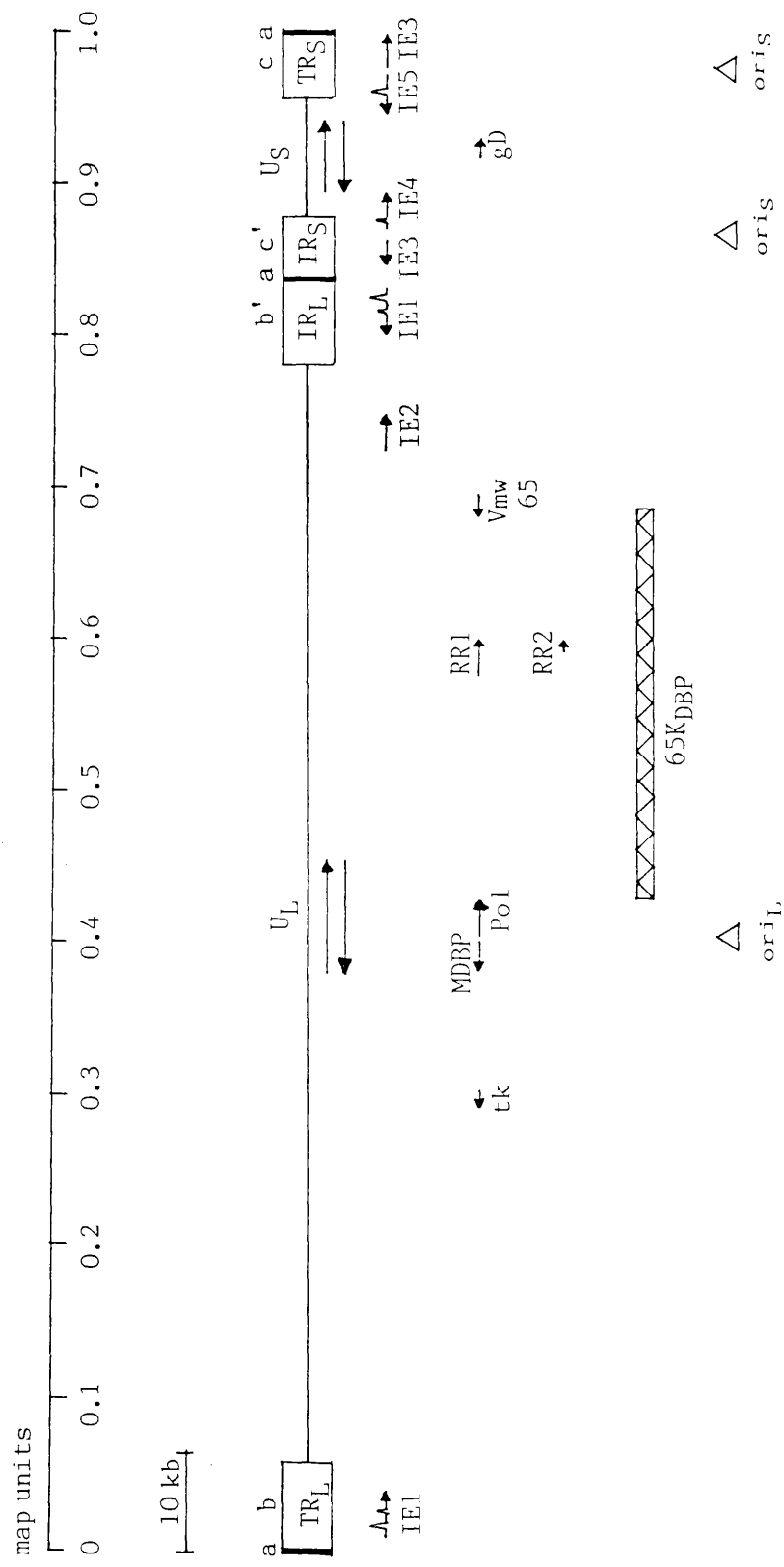
The two serotypes of herpes simplex virus (HSV-1 and HSV-2) belong to the group of Alphaherpesviridae. Characteristics of this group of viruses are a variable host range in vivo and in vitro, a short reproductive cycle in vitro (< 24h), rapid spread of infection in cell culture resulting in mass destruction of susceptible cells and an ability to become latent, most often in ganglia (Roizman, 1982). HSV-1 is generally associated with diseases above the waist, e.g. vesicular lesions of the mouth and lips. HSV-2 is associated with diseases below the waist, e.g. genital lesions.

B1.2STRUCTURE OF THE HSV GENOME

The structure and organization of the HSV genome has recently been reviewed (McGeoch, 1987). Sequencing of the complete HSV-1 genome has been completed (McGeoch et al., personal communication) but the sequence is not yet published.

The HSV genome is a linear, double-stranded DNA molecule. The HSV-1 genome consists of 152 260 bp with a mean G+C content of 68.3% (McGeoch, personal communication). Previously, the G+C content of HSV-1 was estimated to be 67% (Kieff et al., 1971) while the G+C content of HSV-2 was slightly higher at 69% (Goodheart et al., 1968; Halliburton, 1972).

FIG. 1.4 ORGANIZATION OF THE HSV-1 GENOME



The HSV-1 genome is shown to scale in prototype orientation. The long unique ( $U_L$ ) and short unique ( $U_S$ ) regions (single lines) are flanked by a pair of repeat sequences (boxes) in opposing orientation. The terminal and internal short repeats are designated  $TR_S$  and  $IR_S$  respectively. The terminal and internal long repeats are designated  $TR_L$  and  $IR_L$  respectively. A direct repeat sequence, known as the "a" sequence, is present at the genomic termini and in inverted orientation at the L-S junction ( $a'$ ). The sequences which bound the a sequence in  $TR_L$  and  $IR_L$  are termed "b" and "b'" respectively. The sequences in  $TR_S$  and  $IR_S$  which bound the a sequence are termed "c" and "c'" respectively. The four possible isomers of HSV, which differ in the relative orientation of the two unique sequences about the joint, are shown by headed arrows below  $U_L$  and  $U_S$ .

Below the genome are mapped (a) the IE mRNA's (spliced regions are raised), (b) the three HSV-1 origins of DNA replication, (c) Transcripts which specify virus encoded proteins which are specifically mentioned in this thesis.

A full transcript map is not shown. The hatched bar shows the limits for the map location of the 65K<sub>DBP</sub> (DNA binding protein).

Abbreviations and key references are as follows:

|                  |   |
|------------------|---|
| IE               | immediate-early - (IE 1, Perry <u>et al.</u> , 1986; IE 2, Whitton <u>et al.</u> , 1983; IE 3, Rixon <u>et al.</u> , 1982; IE's 4 and 5, Rixon and Clements, 1982). |
| tk               | thymidine kinase (McKnight, 1980)   |
| MDBP             | major DNA binding protein (Quinn and McGeoch, 1985)   |
| Pol              | DNA polymerase (Quinn and McGeoch, 1985)  |
| RR               | ribonucleotide reductase (Frink <u>et al.</u> , 1981; McLauchlan and Clements, 1983).   |
| Vmw 65           | IE <u>trans</u> -acting factor (Dalrymple <u>et al.</u> , 1985)   |
| 65K              | DNA binding protein (Marsden <u>et al.</u> , 1987)  |
| gD               | glycoprotein D (McGeoch <u>et al.</u> , 1985)   |
| oris             | (Stow and McMonagle, 1983)  |
| ori <sub>L</sub> | (Quinn and McGeoch, 1985)   |



The genomes of HSV-1 and HSV-2 are 50% homologous (Kieff et al., 1972) and homologous regions are essentially colinear with viral genes mapping at equivalent positions on both genomes (Preston et al., 1978; Davison and Wilkie, 1983).

As shown in fig. 1.4, the HSV genome consists of two covalently linked components, designated Long(L) and Short(S). Each component contains a unique sequence ( $U_L$  of 110 000 bp and  $U_S$  of 13 000 bp) flanked by a pair of repeat sequences in opposing orientation. The terminal and internal long repeats are designated  $TR_L$  and  $IR_L$  respectively and each consist of 9200 bp. The terminal and internal short repeats, designated  $TR_S$  and  $IR_S$  each consist of 6600 bp. The sequences of  $R_L$  and  $R_S$  are distinct.

A direct repeat sequence of 400 bp, known as the a sequence, is present at the genomic termini and in inverted orientation at the L-S junction(fig. 1.4). The sequences which bound the a sequence in  $TR_L/IR_L$  and  $TR_S/IR_S$  are termed b and c sequences respectively (Davison and Wilkie, 1981).

HSV virion DNA can exist in four isomeric forms which differ in the relative orientation of the two unique sequences about the joint. These isomers appear to be functionally equivalent (Davison and Wilkie, 1983; Longnecker and Roizman, 1986). By convention, one isomer is regarded as the prototype (P), (fig. 1.4). The a sequence is thought to play a role in the inversion of the L and S components (Mocarski and Roizman, 1982; Chou and Roizman, 1985).

The a sequence also plays a role in DNA maturation and encapsidation following DNA replication. Concatameric DNA is processed in the cell nucleus by packaging into nascent nucleocapsids and cutting into genome unit lengths (Vlazny et al., 1982). Signals for cleavage (Varmuza and Smiley, 1985) and encapsidation (Stow et al., 1983) are contained within the a sequence. The order of cutting and packaging is unclear but these two steps are tightly coupled (Deiss and Frenkel, 1986; Stow et al., 1986).

### B1.3            STRUCTURE OF THE VIRION

The herpes virion is 120 - 200 nm in diameter and consists of four distinct morphological elements:

- (a) A central, electron - dense core containing the double-stranded DNA genome (Furlong et al., 1972) is wrapped around an electron - translucent proteinaceous matrix (Furlong et al., 1972; Nazerian, 1974).
- (b) An icosahedral capsid of 100 - 110 nm diameter consisting of 162 capsomeres surrounds the core (Wildy et al., 1960).
- (c) An amorphous, fibrous layer, termed the tegument, appears to be attached to the capsid, probably at the vertices and to the inner surface of the envelope (Roizman and Furlong, 1974; Vernon et al., 1982). The thickness of the tegument varies among different herpesviruses and is genetically determined (McCombs et al., 1971).
- (d) An envelope consisting of a bilayer membrane with spiked projections, approximately 8 nm long, surrounds the tegument (Wildy et al., 1960).

### B1.4            HSV PROTEINS

The subject of the structure and function of HSV proteins is a large one and has been extensively reviewed (Spear, 1985; Dargan, 1986; Marsden, 1987; McGeoch, 1987). A brief summary is given here.

The HSV proteins can be divided into distinct functional groups:

- (a) Structural Proteins include the proteins present in the capsid and tegument of the virion (reviewed by Dargan, 1986) and the

viral glycoproteins which form the spiked projections of the virion envelope, present on the surface of infected cells. The viral glycoproteins are important determinants of viral pathogenicity. They also play a role in adsorption and penetration of the virus into the host cell and possibly also in budding and envelopment of the virus.

(Reviewed by Spear et al., 1985; Marsden, 1987).

- (b) Proteins involved in DNA replication and nucleotide metabolism include thymidine kinase, ribonucleotide reductase, deoxyuridine triphosphatase, DNA polymerase, exonuclease, topoisomerase, an "ori<sub>S</sub>" binding protein and the major DNA binding protein (reviewed by McGeoch, 1987).
- (c) Proteins involved in the regulation of transcription (discussed in section B1.6)
- (d) Other proteins include a novel virus coded protein kinase encoded by the U<sub>S</sub>3 gene that is not required for virus growth in cultured cells (Purves et al., 1986; Purves et al., 1987).

#### B1.5      HSV DNA REPLICATION

The mechanism by which HSV DNA is replicated is not well understood. Present knowledge has recently been reviewed (McGeoch, 1987).

Evidence from electron microscopic studies (Friedmann et al., 1977; Ben-Porat and Rixon, 1979) and from studies on the physical forms of the DNA present throughout the replicative cycle (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981) suggests a rolling circle mechanism which yields large head-to-tail concatameric intermediates that are subsequently cleaved to generate unit-length molecules (Deiss and Frenkel, 1986).

An origin of replication, termed "ori<sub>S</sub>", has been localized (Stow and

McMonagle, 1983) to a 90 bp region of  $R_S$  (thus there are two copies of ori<sub>S</sub>, one in  $TR_S$  and one in  $IR_S$ ). The essential features of ori<sub>S</sub> are the presence of an imperfect palindrome with each arm consisting of 21 residues and a sequence,  $(AT)_6$ , at the centre of the palindrome which is essential for ori function (Stow, 1985). Two copies of the ori<sub>S</sub> sequence have been found within almost identical direct repeats of 137 bp in the  $R_S$  region of HSV-2 (Whitton and Clements, 1984). One, but not two, of the ori<sub>S</sub> sequences is dispensable for growth in culture (Longnecker and Roizman, 1986).

An ori<sub>S</sub> binding protein has been detected in nuclear extracts of infected cells (Elias *et al.*, 1986). The DNase I footprint of this protein spans 18 of the 90 bp minimal ori<sub>S</sub> sequence. Its potential function in DNA replication has not yet been determined. The ori<sub>S</sub> sequence lies within an open reading frame with the potential of coding for a protein of 330 amino acids (Hubenthal-Voss *et al.*, 1987). The functional significance of this is unknown.

Another origin of replication, termed "ori<sub>L</sub>", is located within a 296 bp region near the centre of  $U_L$  (Gray and Kaerner, 1984) situated between two genes encoding important proteins for DNA replication; DNA polymerase and the major DNA binding protein (fig. 1.4). The ori<sub>L</sub> sequence contains a long perfect palindrome with arms of 72 residues, showing striking homology to ori<sub>S</sub> (Quinn and McGeoch, 1985; Weller *et al.*, 1985). Additional symmetry elements include short sequences forming an "inner" interrupted inverted repeat within each arm of the large palindrome. As for ori<sub>S</sub>, an A+T-rich region is situated at the centre of the palindrome. Ori<sub>L</sub> is dispensable without affecting viral growth (Orberg and Schaffer, 1987) or viral replication *in vitro* (Polvino-Bodnar *et al.*, 1987).

Seven HSV genes necessary for transient replication of plasmids containing ori<sub>S</sub> or ori<sub>L</sub> have been identified (Wu *et al.*, 1988) and sequenced (McGeoch *et al.*, 1988), two of which encode the viral DNA polymerase and a single-stranded DNA-binding protein.

B1.6.1 Temporal Regulation of Gene Expression

Infection of permissive cells with HSV results in the expression of three temporally regulated sets of viral genes termed immediate-early (IE or  $\alpha$ ), early (E or  $\beta$ ) and late (L or  $\gamma$ ) that are expressed in a coordinately regulated manner (Honess and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). Transcription of HSV DNA is dependent on the host RNA polymerase II (Costanzo et al., 1977).

IE genes are expressed immediately after virus infection, independently of de novo protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). Expression of the later classes of genes is dependent on the prior synthesis of IE gene products (Honess and Roizman, 1974, 1975; Preston, 1979a; Watson and Clements, 1980).

E mRNA's are detectable by 2h post-adsorption (p.a.) and reach maximum levels by about 4 - 6 h (Wagner, 1985). The E or  $\beta$  genes can be divided into two subgroups,  $\beta_1$  and  $\beta_2$ , depending on the time of their expression (Mavromara-Nazos et al., 1986).

L genes are maximally expressed after the onset of DNA replication (initiated at about 2h p.a.) and reach maximum levels by 10 - 16 h p.a. (Holland et al., 1980; Wagner, 1985).

The late genes can be divided into two groups:

In the absence of DNA replication, the early-late (EL or  $\gamma_1$ ) transcripts are detectable in the cytoplasm although DNA replication is required for maximum expression. Transcription of true-late (TL or  $\gamma_2$ ) genes has an absolute requirement for viral DNA replication (Holland et al., 1980; Wagner, 1985).

### Bl.6.2      HSV IE Gene Products

The HSV genome codes for five IE gene products which are named according to their mobility on SDS-PAGE gels in terms of apparent mol. wt. ( $V_{mw}$ ), (Preston et al., 1978; Watson et al., 1979) or by "infectious cell polypeptide" (ICP) number (Honess and Roizman, 1974; Morse et al., 1978).

| <u>Gene Number</u> | <u>Apparent Mol. Wt.</u> | <u>ICP Number</u> |
|--------------------|--------------------------|-------------------|
| IE 1               | $V_{mw}$ 110             | ICP 0             |
| IE 2               | $V_{mw}$ 63              | ICP 27            |
| IE 3               | $V_{mw}$ 175             | ICP 4             |
| IE 4               | $V_{mw}$ 68              | ICP 22            |
| IE 5               | $V_{mw}$ 12              | ICP 47            |

The IE mRNA's have been mapped on the HSV genome (fig. 1.4), (Clements et al., 1979; Watson et al., 1979; Anderson et al., 1980; Rixon and Clements, 1982; Rixon et al., 1982; Whitton et al., 1983; Rixon and McGeoch, 1985; Perry et al., 1986).

### Bl.6.3      Regulation of IE Gene Expression: Role of the Virion TIF

Regulation of IE gene expression has been extensively studied by biochemical and genetic methods. The biochemical approach has involved the use of chimaeric plasmids containing the 5'-flanking sequences of an IE gene fused to the structural gene sequence of the E gene, thymidine kinase (tk). Several assay systems have been used to study the expression of the tk gene in these constructs:

- (a) Recombination of the chimaeric plasmids into the virus genome and measurement of tk activity and RNA levels.
- (b) The ability of these plasmids to convert  $tk^-$  cells to the  $tk^+$  phenotype.

- (c) Microinjection of Xenopus oocytes with chimaeric plasmids and measurement of tk activity and RNA levels.
- (d) Transfection of tissue culture cells with chimaeric plasmids and measurement of tk activity and RNA levels.

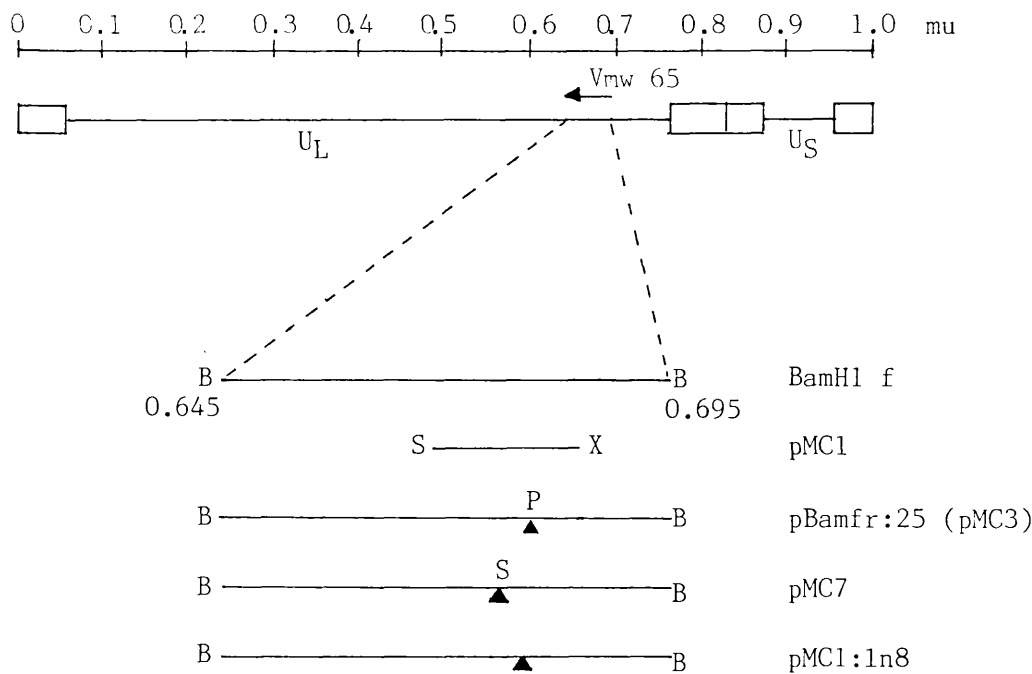
Regulatory sequences for IE gene expression have been located in the 5'-terminus of the IE mRNA start site. These sequences confer IE regulatory properties to the tk gene in that expression can occur in the absence of de novo protein synthesis and expression is induced in the presence of the HSV virion (Post et al., 1981; Batterson and Roizman, 1983). The virion component responsible for this induction, termed trans-inducing factor (TIF), has been identified by cotransfection experiments (Campbell et al., 1984) as the major late phosphoprotein, Vmw 65, which is assembled into the tegument region of the virion at approximately 400 - 600 molecules per particle (Heine et al., 1974). The gene for Vmw 65 maps within the Bam H1 fragment on the HSV-1 genome (fig. 1.5) and has been sequenced (Dalrymple et al., 1985; Pellet et al., 1985).

The IE gene 5'-flanking sequences have been investigated in more detail by sequence analysis and by expression of chimaeric plasmids containing specific regions or deletions of the 5'-flanking sequences linked to the tk gene. From analysis of the promoter/regulatory regions of IE genes 1 and 2 (Mackem and Roizman, 1982b; Kristie and Roizman, 1984), IE gene 3 (Mackem and Roizman, 1982a; Murchie and McGeoch, 1982; Cordingley et al., 1983; Lang et al., 1984; Bzik and Preston, 1986) and IE genes 4/5 (Murchie and McGeoch, 1982; Preston et al., 1984), several cis-acting regulatory signals have been identified:

- (a) A "TATA" homology located approximately -25 bp 5' to the start of IE mRNA transcription.
- (b) Multiple binding sites for the general transcription factor, Spl (Jones and Tjian, 1985).
- (c) Sequences homologous to the core (GTGG<sup>AAA</sup><sub>TTT</sub>G) of the SV40 enhancer (Weiher et al., 1983).

FIG. 1.5

LOCATION OF  $V_{mw}$  65 CODING SEQUENCE ON THE HSV-1 GENOME  
AND DESCRIPTION OF PLASMIDS USED IN TRANSFECTION EXPERIMENTS



(Adapted from Campbell et al., 1984 and Dalrymple et al., 1985).

A diagrammatic representation of the HSV-1 prototype genome.  $U_L$  and  $U_S$  are the long and short unique regions of the genome. The open boxes represent repeated sequences. Restriction endonuclease cleavage sites are abbreviated as follows: B, Bam HI; P, Pvu  $\overline{\text{II}}$ ; S, Sal  $\overline{\text{I}}$ ; X, Xho  $\overline{\text{I}}$ . BamH1 f is located between 0.645 and 0.695 map units. The coding sequence for  $V_{mw}$  65 is contained entirely within the Sal  $\overline{\text{I}}$  / Xho  $\overline{\text{I}}$  restriction fragment of BamH1 f (Dalrymple et al., 1985). The plasmid pMC1 contains the Sal  $\overline{\text{I}}$  / Xho  $\overline{\text{I}}$  fragment cloned into the vector pAT 153 (as described by Campbell et al., 1984). The plasmids pBamfr:25 (pMC3), pMC7 and pMC1:ln8 contain the BamH1 f fragment with various insertion mutations (represented as ▲), as described in section 6.8.2.



- (d) Far-upstream sequences (up to -340 bp) containing at least one copy of the A+T-rich consensus sequence TAATGARAT (where R = purine), required for regulation of transcription by the virion TIF.

The far-upstream region shows enhancer - type properties (Lang et al., 1984; Preston and Tannahill, 1984). Expression of the tk gene is enhanced when the orientation of this region is reversed with respect to the promoter and when its distance from the promoter is increased by over 1000 bp. However, some positional constraints do operate as the element works less well when placed downstream of the tk promoter than when placed upstream (Lang et al., 1984; Preston and Tannahill, 1984). Although the TAATGARAT element in one or more copies is sufficient for induction by the TIF (Gaffney et al., 1985; O'Hare and Hayward, 1987), enhancer activity is increased by flanking G+C-rich sequences (consensus CCCGCC or its complement, GGGCGGG) and an upstream G+A-rich element (Bzik and Preston, 1986).

The mechanism of action of the virion TIF is not yet completely understood. Attempts to demonstrate direct binding of the TIF to the promoter/regulatory domains of IE genes and specifically to the TAATGARAT sequence have been unsuccessful. However, recent evidence (Kristie and Roizman, 1987; Preston et al., in press) has shown that cellular proteins present in both infected and uninfected cell nuclei recognize and complex with the the TAATGARAT sequence. This suggests that trans-activation by the TIF, although sequence specific, is not due to direct interaction of the TIF with its target sequence. Instead, a complex is formed with one or more cellular factors which then interacts with TAATGARAT DNA.

Other viral proteins may also be involved in the action of the TIF. McKnight et al. (1987) have shown that two viral proteins, encoded by a region of the HSV-1 genome 3' to the gene encoding the TIF, are able to modulate the activity of the TIF in trans. Whether or not these proteins complex with the TIF is yet to be determined.

#### B1.6.4 Regulation of Gene Expression by IE Proteins

Three of the five IE proteins (Vmw 110, Vmw 175 and Vmw 63) are able to regulate gene expression.

##### B1.6.4.1 Vmw 110

The product of IE gene 1, Vmw 110, is a phosphorylated polypeptide which is synthesized early in infection and rapidly translocated to the nucleus (Pereira et al., 1977; Ackermann et al., 1984). Vmw 110 is bound to chromatin in infected cell nuclei (Hay and Hay, 1980) but there is no direct evidence for binding of Vmw 110 to DNA.

Cotransfection experiments have shown that Vmw 110 is able to activate gene expression from all three major classes of HSV promoters (Everett, 1984a, 1986; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a, 1985b; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986). The activation of E promoters by Vmw 110 is increased in the presence of Vmw 175 (Everett, 1984a; 1986; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986). In the case of IE gene expression, repression by Vmw 175 (section B1.6.4.3) is dominant over activation by Vmw 110 (O'Hare and Hayward, 1985b). Sequences upstream of IE genes responding in trans to Vmw 65, Vmw 110 and Vmw 175 are distinct (Gelman and Silverstein, 1987a; O'Hare and Hayward, 1987).

The combination of Vmw 110 and Vmw 175 can activate plasmid borne cellular promoters (Everett, 1985). It has been suggested (O'Hare and Hayward 1985b) that Vmw 110 trans-activates genes non-specifically since it can activate each class of HSV promoter as well as heterologous promoters. Trans-activation may occur by recognition of, or interaction with, some signal which is common to many promoter/regulatory regions such as the TATA homology and short G+C-rich domains.

The complete DNA sequence and mapping of the RNA of IE gene 1 (Perry et al., 1986) has been used as a basis for detailed mutational analysis

of Vmw 110 (Everett, 1987) to define functional domains of the protein. No mutations affected nuclear localization but at least five regions of Vmw 110 were important for trans-activation of gene expression. The precise role of each of these mutation sensitive regions is not yet known.

The role of Vmw 110 in lytic infection is unclear. Studies with deletion and ts mutants of IE gene 3 (DeLuca et al., 1985) have shown that the presence of functional Vmw 110, in the absence of functional Vmw 175, is not sufficient to induce expression of E and L genes. Moreover, in cell lines carrying the anti-sense mRNA for Vmw 110 (Sandri-Goldin et al., 1987), E and L gene expression is not significantly affected by the lower concentration of Vmw 110. Viruses in which the majority of the IE gene 1 coding region is deleted (Stow and Stow, 1986; Sacks and Schaffer, 1987) are able to grow in tissue culture but plaque relatively inefficiently. Stow and Stow (1986) have suggested that the mutant exhibits a defect in initiation of a productive infection at low multiplicity which can be overcome by increasing the multiplicity of infection. Thus Vmw 110 does not seem to be essential for productive infection in cell culture but is required for fully efficient viral growth.

#### B1.6.4.2 Vmw 63

The product of IE gene 2, Vmw 63, is a phosphorylated, nuclear protein (Wilcox et al., 1980). Genetic and phenotypic characteristics of four ts mutants in IE gene 2 (Sacks et al., 1985) indicate a regulatory role for Vmw 63 in viral gene expression. The mutants overproduce certain IE polypeptides at the non-permissive temperature (N.P.T.) and fail to produce a number of L proteins. Since a L function has been implicated in negative regulation of IE genes (DeLuca et al., 1984), it may be that this factor is not synthesized by the ts mutants of Vmw 63 at the N.P.T. resulting in the observed overproduction of certain IE polypeptides. The ts mutants are all able to synthesize E proteins and to induce significant levels of

DNA synthesis. Therefore, Vmw 63 does not seem to be required for transition from IE to E gene expression or for viral DNA synthesis. This result is supported by the inability of plasmid encoded copies of Vmw 63 to stimulate E gene expression in transient assays (Everett, 1984a; DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a). Vmw 63 may play a role in L gene expression since some of the ts mutants in IE gene 2 (Sacks et al., 1985) failed to produce certain L proteins at the N.P.T. This result is supported by the ability of Vmw 63 to activate (in combination with Vmw 175 and Vmw 110) the late VP5 promoter (Everett, 1986).

#### B1.6.4.3 Vmw 175

Vmw 175, the product of IE gene 3, is a phosphorylated polypeptide which is synthesized early in infection and rapidly translocated to the nucleus (Pereira et al., 1977; Wilcox et al., 1980). The distribution of Vmw 175 within the nucleus at E and L times varies (Knipe et al., 1987) suggesting that stimulation of transcription by Vmw 175 may involve different molecular events or contacts during these two periods of the replicative cycle. When extracted with high salt from infected cell nuclei, the native protein is a homodimer (Metzler and Wilcox, 1985).

A number of ts mutants have been described which overproduce IE mRNA's and fail to produce E and L mRNA's at the N.P.T. (Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980). Infection by these ts mutants at the permissive temperature results in a similar pattern of mRNA and protein synthesis as with wild-type virus. All of these mutants map in IE gene 3 (Dixon and Schaffer, 1980; Preston, 1981). The mutant, tsK produces an abnormal Vmw 175 protein at the N.P.T. which is not properly processed to forms of lower electrophoretic mobility and is altered in its subcellular localization (Preston, 1979b). Overall, the studies with ts mutants have shown that Vmw 175 is involved in the transition from IE to E protein synthesis and is required continuously to maintain E and L protein synthesis.

Studies with HSV-1 deletion mutants of Vmw 175 (DeLuca et al., 1985) have confirmed and extended the results obtained with ts mutants. In the absence of functional Vmw 175, significant levels of E genes are not expressed. The phenotypes of the deletion mutants indicate that the exaggerated levels of IE polypeptides induced by ts mutants of IE gene 3 are due, in part, to activities associated with the ts polypeptides themselves. Studies with HSV-2 deletion mutants of Vmw 175 (Smith and Schaffer, 1987a) and with intertypic recombinants of HSV-1 and HSV-2 Vmw 175 (Smith and Schaffer, 1987b) have shown that the HSV-1 and HSV-2 proteins are functionally interchangeable despite a number of biochemical differences.

Transfection experiments (O'Hare and Hayward, 1985b, 1987; Gelman and Silverstein, 1987a) have shown that Vmw 175 inhibits transcription from its own promoter but activates transcription from E promoters. Inhibition of transcription from the IE promoter by Vmw 175 is dominant over activation by Vmw 110 or Vmw 65 and the responsive sequences are distinct (Gelman and Silverstein, 1987a; O'Hare and Hayward, 1987). The inhibition of IE gene expression and activation of later classes of genes has been confirmed by in vitro transcription studies (Beard et al., 1986; Pizer et al., 1986) using cloned HSV genes and nuclear extracts prepared from infected and uninfected HeLa cells. The E protein, ICP 8, also plays a role in shutting off Vmw 175 expression at late times post-infection (Godowski and Knipe, 1986). Different functions of Vmw 175 may reside on different domains of the protein.

Binding sites for partially purified Vmw 175 have been identified by DNase I footprinting in the HSV glycoprotein D (gD) promoter and in the tet<sup>R</sup> gene of the vector pBR322 (Faber and Wilcox, 1986). The consensus binding site sequence is 5'-ATCGTCNNNNYCGRC-3' (N = any base, Y = pyrimidine, R = purine). Gel retardation assays, using HeLa nuclear cell extracts and incorporating monoclonal antibodies to Vmw 175, have been used to demonstrate binding of Vmw 175 to the promoter regions of IE genes (Kristie and Roizman, 1986a, 1986b;

Muller, 1987). The results of Kristie and Roizman (1986b) showed binding of Vmw 175 within a 59 bp sequence taken from the regulatory region of IE gene 3. Deletions within this 59 bp sequence, including removal of Spl binding sites, did not affect binding. The Faber and Wilcox (1986) Vmw 175 consensus sequence is not present in the 59 bp sequence. In contrast, Muller (1987) detected binding of Vmw 175 to the sequence -10 to +3 of IE gene 3. This sequence contains the Faber and Wilcox consensus. The reason for the discrepancy between the results of Kristie and Roizman and those of Muller is unclear. However, the results of Muller support the results of transfection studies by O'Hare and Hayward (1987) who found that the region from -108 to +30 relative to the IE gene 3 mRNA start site mediates its repression. It is not known if cellular proteins are involved in the mechanism of action of Vmw 175.

#### B1.6.5 Regulation of Early Gene Expression

As already discussed (section B1.6.3), the regulatory cis- and trans-acting factors involved in IE gene expression have been clearly defined. In contrast, the factors which distinguish E and L genes are less distinct. Expression of the E genes is dependent on the prior expression of IE proteins (Honess and Roizman, 1974; 1975; Preston, 1979a; Watson and Clements, 1980).

##### B1.6.5.1 Structure of the E Promoter

Functional analysis of the upstream regulatory sequences of the E genes, gD (Everett, 1983; 1984b) and tk (McKnight et al., 1981; McKnight and Kingsbury, 1982; McKnight et al., 1984; Eisenberg et al., 1985; El Kareh et al., 1985) has shown that full trans-activation of these promoters by viral proteins requires 83 bp and 109 bp of sequence 5' to the gD and tk mRNA start sites respectively. Sequence comparison of the E promoters gD, tk and ICP 8 (Su and Knipe, 1987) has identified the following common promoter elements:

- (a) "TATA" homology about -25 bp 5' to the mRNA start of transcription.
- (b) Distal upstream promoter region containing A+C-rich sequences and G+C-rich inverted repeat sequences.
- (c) "CCAAT" homology positioned about -80 in the tk but not in the ICP 8 or gD promoters.

The distal upstream element is able to potentiate transcription, albeit with different efficiencies, when inverted or positioned at variable distances relative to the TATA box (Everett, 1984b, McKnight et al., 1984).

The SV40 E promoter contains six tandem G+C-rich direct repeated sequences, each of which bind the Spl cellular transcription factor resulting in activation of SV40 early RNA synthesis (Dylan and Tjian, 1983a; 1983b). The two distal G+C-rich hexanucleotides (at -100 and -50) of the tk promoter also bind Spl (Jones et al., 1985; McKnight and Tjian, 1986), as do G+C-rich sequences present in HSV IE promoters and a variety of other eukaryotic promoters (Jones and Tjian, 1985; McKnight and Tjian, 1986).

Another general transcription factor, termed "CCAAT transcription factor" (CTF) or "CCAAT binding factor" (CBF), binds to the CCAAT sequence present at about -80 in the tk promoter (Jones et al., 1985; Graves et al., 1986).

Thus no sequence elements unique to HSV E promoters have been identified. The general, cellular transcription factors may cooperate with viral proteins to form an activating complex at the promoters of E genes or the trans-acting factors may each function separately to change the chromatin to an "open" configuration to allow transcription to occur.

## B1.6.6      Regulation of Late Gene Expression

Late genes have been operationally defined as those genes whose expression is dependent on viral DNA replication (Jones and Roizman, 1979; Holland et al., 1980) although some late genes (EL) are expressed in the absence of DNA replication (section B1.6.1).

Expression of Vmw 175 is necessary for late gene expression (DeLuca and Schaffer, 1985; Godowski and Knipe, 1986). There is some evidence that the E protein, ICP 8, is involved in negative regulation of late gene expression (Godowski and Knight, 1985).

### B1.6.6.1    Structure of the L Promoter

Experiments with chimaeric plasmids (Silver and Roizman, 1985; Mavromara-Nazos et al., 1986) containing E or L upstream sequences linked to the coding sequence of the HSV tk gene have shown that L genes are differentially regulated depending on their genomic environment. When integrated into the host cell genome, E and L genes could not be distinguished. In contrast, when resident in the viral genome, the chimaeric L-tk gene was regulated as a L gene, requiring DNA synthesis for expression. Similar results were obtained by Costa et al. (1985) who studied the differential regulation of an E and L gene in a transient assay system. Promoter sequence was not sufficient to distinguish between E and L genes in these assays (Costa et al., 1985).

Experiments with plasmid constructs containing the promoter of the L gene U<sub>S</sub>11 linked to the coding sequence of the rabbit  $\beta$ -globin gene with or without an HSV-1 origin of replication (ori<sub>S</sub>) have shown that, in the presence of a functional origin of replication, all the DNA sequence requirements for efficient expression of the L gene are contained within 31 bp of the RNA cap site (Johnson and Everett, 1986a; 1986b). A functional origin of replication is sufficient to convert an E promoter into a L promoter (Johnson and Everett, 1986b). Another L gene (gC) has similar sequence requirements (-34 to +124) for efficient expression (Homa et al., 1986).



B1.7.1 The "Shutoff" Effect

Infection of cultured cells with HSV-1 or HSV-2 results in rapid inhibition of host DNA synthesis (Roizman and Roane, 1964), RNA and protein synthesis (Roizman et al., 1965; Sydiskis and Roizman, 1966). Host polysomes disaggregate (Sydiskis and Roizman, 1966, 1967) and new polysomes contain mainly virus encoded mRNA's (Stringer et al., 1977).

Understanding the events involved in host shutoff has been complicated by the variability of response seen with different virus strains and cell types. HSV-1 (F) infection of Friend erythroleukaemia cells results in a rapid shutoff of globin synthesis and mRNA production, (Nishioka and Silverstein, 1977, 1978) while histone mRNA is selectively stabilized (Mayman and Nishioka, 1985). However, infection of Vero cells with HSV-1 (F, KOS and 17+) or HSV-2 (G) results in degradation of histone H3 mRNA along with actin mRNA (Shek and Bachenheimer, 1985). Infection of the methotrexate resistant cell line KB/6b with HSV-2 (333) causes a rapid reduction in dihydro-folate reductase synthesis and mRNA production (Bastow et al., 1986) whereas HSV-2 (HG 52) has no effect on cellular mRNA.

Studies by Nishioka and Silverstein (1977, 1978) and by Fenwick and Clark (1982) suggest that early shutoff of host protein synthesis is due to polysome disaggregation mediated by a virion component while delayed shutoff, involving increased catabolism of cellular mRNA, is dependent on viral gene expression. Studies by other groups (Inglis, 1982; Read and Frenkel, 1983; Fenwick and McMenamin, 1984; Shek and Bachenheimer, 1985; Bastow et al., 1986) have demonstrated that rapid degradation of certain mRNA's is an early event induced by a virion - associated factor.

A number of virion host shutoff (vhs) mutants of HSV-1 (KOS) have been described (Read and Frenkel, 1983) which are defective in the

function responsible for the initial suppression of host protein synthesis. Further studies by Strom and Frenkel (1987) have shown that vhs mutants do not degrade host mRNA to the same extent as wild-type virus. The extent of mRNA degradation correlates well with the extent of inhibition of host protein synthesis, suggesting that inhibition of host protein synthesis and degradation of host mRNA are mediated by the same virion - associated function.

Preliminary characterization of the mutant, vhs-1 (Read and Frenkel, 1983) revealed a defect in post-transcriptional shutoff of HSV IE protein synthesis. Infection with vhs-1 results in increased stability of at least three different mRNA's (from IE genes -1, -2 and -3) compared to the instability of these IE mRNA's during wild-type infection (Oroskar and Read, 1987). Purified virions of the wild-type virus, but not of the vhs-1 mutant virus, carry a function that reduces the half-life of host and IE mRNA's (Kwong and Frenkel, 1987). Thus the wild-type virus carries a function that is able to reduce the half-life of both host and viral transcripts. Early translational shutoff may be a consequence of this function.

#### B1.7.2      Activation of Cellular Genes

Certain cellular polypeptides are expressed at elevated levels as a result of HSV infection. Heat shock or stress proteins are induced in chicken embryo fibroblast cells by infection with tsK (section B1.6.4.3) at the N.P.T. (Notarianni and Preston, 1982). In this case, the stress response is due to the presence of abnormal forms of Vmw 175 rather than the overproduction of IE genes (Russell et al., 1987). Natural isolates of HSV-1 frequently possess mutations in the gene coding for Vmw 175 (Knipe et al., 1981; Post et al., 1981). Thus induction of the stress response by HSV may be important during infection in vivo. Virus induction of heat shock genes occurs with adenovirus (Nevins, 1982; Kao and Nevins, 1983), polyoma virus and SV40 (Khandjian and Turler, 1983) as well as with the oncogene, c-myc (Kingston et al., 1984).

HSV infection of rat embryo cells results in the accumulation of certain cellular polypeptides (including proteins of mol. wt. 90 000 and 40 000), (Macnab et al., 1985). These proteins are highly expressed in cells transformed by HSV and by other DNA or RNA viruses and in immortalised cell lines but not in primary cells. Infection of human embryo fibroblast cells with HSV-2 induces the synthesis of a minor cellular stress protein of mol. wt. 57 000 which is growth regulated (La Thangue et al., 1984) and transcriptionally activated (Patel et al., 1986). Transcriptional activation of a cellular gene has also been shown by Kemp et al. (1986).

Several herpesviruses (including HSV-1 and HSV-2) and adenovirus-2 are able to activate plasmid borne viral and cellular promoters during infection (Everett and Dunlop, 1984). In addition,  $\epsilon$ - and  $\beta$ -globin promoters integrated into biochemically transformed cell lines are activated by HSV-1 infection and by transfection of the IE genes, Vmw 175 and Vmw 110 (Everett, 1985). However, the endogenous  $\beta$ -globin gene of rabbit kidney cells is not activated by virus infection (Everett, 1985). The author suggests that this may be due to the chromatin configuration of the promoter region of the endogenous globin gene. Many host promoters may be in regions of densely packed chromatin and so would not be readily available for activation.

C1                    ROLE OF STEROID HORMONES AND VIRUS IN THE DEVELOPMENT  
AND PATHOLOGY OF THE UTERINE CERVIX.

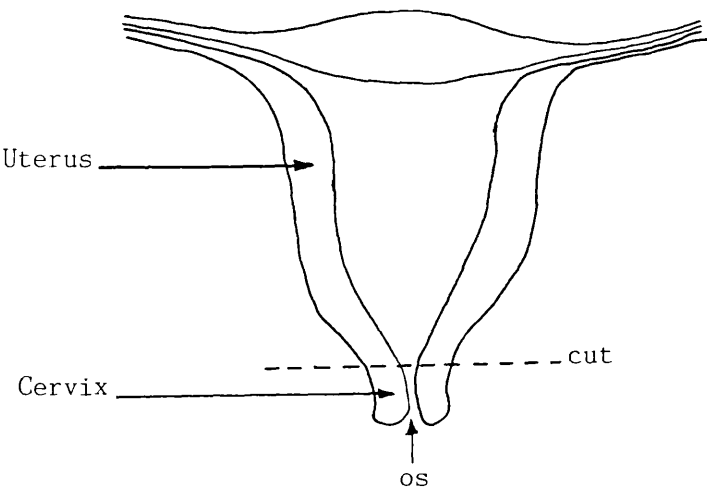
C1.1                ANATOMY AND PATHOLOGY OF THE CERVIX: GENERAL DESCRIPTION

C1.1.1            Anatomy

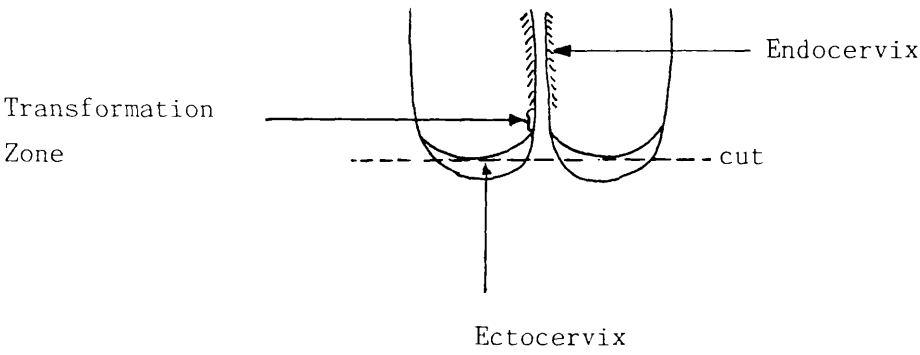
The uterine cervix is a fibromuscular tube connecting with the uterine cavity at the internal os and opening out into the vagina at the external os (fig. 1.6). The surface of the tubular portion is called the endocervix and is lined by columnar epithelium. These cells secrete mucus. The part of the cervix exposed to the vagina is called the ectocervix and consists of keratinising, stratified, squamous epithelium. There is an abrupt change at the external os from the columnar to the squamous type of epithelium. This junction can change position due to a process known as squamous metaplasia which occurs particularly at the time of menarche and also in response to oral contraception and pregnancy. During this process, columnar epithelium migrates, perhaps initiated by oestrogenic stimulation and, under the influence of the acidic environment of the vagina, squamous epithelium forms. Exactly how this happens is unknown.

The area involved in squamous metaplasia is known as the transformation zone. Squamous cancer of the cervix arises almost invariably from the transformation zone. This zone contains numerous cells in premitotic or mitotic phases of the cell cycle. Such cells are perhaps more susceptible to cancerous transformation than quiescent epithelial cells. 90 - 95% of all cervical cancers arise from the squamous epithelium whereas 5 - 10% arise from columnar epithelium (adenocarcinoma). A small number of cancers are of a mixed type (adenosquamous carcinoma).

FIG. 1.6      ANATOMY AND DISSECTION OF THE UTERINE CERVIX



Excision of ectocervix



### Cl.1.1.2      Definition of Cervical Intraepithelial Neoplasia

Development of cervical cancer progresses through a series of graded precancerous lesions. In the normal squamous epithelial covering of the vagina and ectocervix there is a single layer of basal cells, separated from the underlying stroma by the basement membrane. Above the basal layer there are three or four layers of parabasal cells which display intense mitotic activity. Dysplastic lesions of the parabasal layer are graded into cervical intra-epithelial neoplasia (CIN) grade I, grade II and grade III (which corresponds to carcinoma in situ or CIS, where the whole epithelium shows dysplasia). Invasive carcinoma is when the basement membrane is broken.

### Cl.1.1.3      Aetiology and Epidemiology of Cervical Cancer

Development of cervical cancer is a multifactorial process. As a result of investigations into various cultural, ethnic and social variables of women with premalignant and malignant lesions of the cervix, a number of high risk factors have been identified including multiple sexual partners and early age of first intercourse (Rotkin, 1973; Berg and Lampe, 1981; Graham et al., 1982) leading to the idea that cervical cancer is venereally transmitted (discussed in sections Cl.3 and Cl.4). Other non-sexual variables include cigarette smoking (Wigle, 1980; Trevathan et al., 1983) and long-term exposure to the contraceptive pill (Harris et al., 1980). Exposure to the non-steroidal oestrogen, diethylstilboestrol (DES) is associated with development of clear cell adenocarcinoma (section Cl.2.4).

The following sections describe the role of hormones in the normal growth and development of the cervix, the role of hormones and viruses in the development of cervical cancer and the possible interaction of hormones and viruses in transformation of cells.

Cl.2            ROLE OF STEROID HORMONES IN THE NORMAL DEVELOPMENT,  
FUNCTION AND PATHOLOGY OF THE CERVIX.

Cl.2.1        Changes in Cervical Secretions During the Menstrual Cycle

The secretory activity of the cervical epithelium is governed by hormonal changes (reviewed by Lunenfeld and Insler, 1978). The daily production of mucus varies between 20 mg per day at the beginning of the cycle and 600 mg per day at mid-cycle. When under strong oestrogenic stimulation (i.e. during the ovulatory phase of the menstrual cycle or during oestrogen treatment), the uterine cervix produces abundant, watery, thin mucus due to the increased water and electrolyte content. Sperm permeability during this period is at its highest. When under the influence of both oestrogen and progesterone (i.e. during the luteal phase of the menstrual cycle, under combined contraceptive pill or in pregnancy) the cervical epithelium produces scant, thick mucus of great viscosity which forms a barrier to sperm.

Cl.2.2        Ripening of the Cervix During Pregnancy

Pregnancy induces a number of biochemical changes in cervical stroma (Calder, 1981). The cervix becomes increasingly softer (due to changes in collagen) and more vascular. The shape of the cervix changes due to alterations in cervical length and diameter. Ripening of the cervix is controlled by complex endocrine mechanisms.

Cl.2.3        Repair of Cervical Erosions

Hormones influence the processes of regeneration and preneoplastic transformation which occur during the re-epithelialization of cervical erosions. Oestrogens stimulate the proliferation of the stratified squamous epithelium of the ectocervix but not the columnar epithelium

of the endocervix. Progesterone stimulates the columnar epithelium and reserve cells beneath it but not the squamous epithelium. In a small number of cases, re-epithelialization does not proceed smoothly to normal maturation of the overgrowing epithelium. Instead, pre-cancerous lesions of various grades may form. In such cases, the prolonged increase in proliferative activity may prepare the way for carcinogenic substances to manifest their effects.

#### Cl.2.4      Effect of In Utero Exposure to DES

The non-steroidal oestrogen, diethylstilboestrol (DES), used in the 1950's to prevent abortion and other complications during pregnancy (Smith, 1948) has been reported to be responsible for the development of clear cell adenocarcinoma of the vagina and cervix in female offspring at menarche (Herbst et al., 1971; Noller et al., 1972). Further, several non-malignant epithelial lesions have also been observed in the lower genital tract (Robboy et al., 1978) in females exposed to DES in utero. Major investigations of the frequency and natural history of genital tract lesions in such women (Lebarthe et al., 1978; Robboy et al., 1979; Herbst, 1981) have shown that vaginal epithelial changes are related to a number of variables including total dosage, duration of exposure and time in the menstrual cycle of first exposure. Risk factors such as sexual behaviour were difficult to account for. Almost all cases of CIS or marked grades of dysplasia were from the external os or around the transformation zone suggesting that these lesions may be coincidental rather than related to DES exposure. No case of invasive squamous carcinoma was found. However, there did appear to be a link between exposure to DES and the development of clear cell adenocarcinoma.

#### Cl.2.5      Mouse Model for the Role of Hormones in Cervical Cancer

Some studies have been done on the effect of sex steroid hormones on the mouse genital tract. Neonatal administration of oestrogen, progesterone or DES in the mouse leads to abnormalities in the vagina



and cervix and, in some cases, the development of lesions with histological features resembling squamous cell carcinoma (Takasugi, 1976; Jones and Bern, 1979; McLachlan et al., 1980). Transplantation of cervicovaginal tissue of mice neonatally treated with oestrogen and/or progesterone into syngeneic hosts resulted in six tumours within six months while transplantation of control cervicovaginal tracts did not give rise to tumours (Jones and Pacillas-Verjan, 1979).

Some questions have been raised as to the validity of the mouse model for studying cervical cancer (Muñoz, 1976). Cervical cancer is extremely rare in untreated mice but relatively frequent in women. Transition from dysplasia to CIS is also rare in mice and more frequent in women. The cancers in both species are squamous cell carcinomas but cancers in mice tend to be well differentiated with limited infiltration and few metastases whereas in women, the tumours are usually undifferentiated, highly malignant and, if untreated, give rise to extensive distant metastases.

#### Cl.2.6      Steroid Receptors in Cervical Tissue

Very few studies on ER and PR levels in normal cervix and cervical tumour tissue have been carried out. The current state of knowledge has recently been reviewed (Soutter and Leake, 1987). Studies by Sanborn et al. (1975, 1978) have demonstrated the presence of cytoplasmic ER and PR in the various tissue components of the cervix. The level of cytoplasmic ER was uninfluenced by hormonal changes during the menstrual cycle although cytoplasmic PR was depressed during the luteal phase of the cycle. Soutter et al. (1981, 1983) found both cytoplasmic and nuclear ER in all samples of normal premenopausal cervical tissue assayed. The level of receptor in cervix is lower than in the more recognized hormone dependent tissues such as breast and endometrium and in some studies only a small proportion of normal cervical tissue samples were ER positive (Toppila et al., 1983).

Several studies have shown the presence of cytoplasmic ER's in squamous cervical cancer. However, estimates of the proportion of cervical tumours which contain ER's vary enormously (reviewed by Soutter and Leake, 1987). Only one group has reported on nuclear ER's (Soutter et al., 1981, 1983). In these studies, low concentrations of nuclear ER were detected in 22.4% of tumours, almost always in conjunction with cytoplasmic ER. However, more recent studies on tissue stored under better conditions shows nuclear ER in 45.2% of 73 squamous tumours (Soutter and Leake, 1987). The presence of cytoplasmic PR has also been demonstrated. Thus it seems that at least a proportion of squamous cervical tumours contain a functional ER system which could prove useful in disease prognosis and in treatments.

Although less data is available for adenocarcinoma of the cervix, there seems to be better agreement among studies with approximately 45 - 65 % of adenocarcinomas showing the presence of functional receptors (Soutter and Leake, 1987).

The usefulness of receptor status in cervical cancer is uncertain as the receptor content of cervical tumours seems to bear no relationship to menopausal status or the stage of the disease or degree of differentiation of the tumour (Soutter and Leake, 1987).

### C1.3        ASSOCIATION OF HSV WITH CERVICAL CANCER

#### C1.3.1      Association of HSV with Human Cervical Tumours

For many years, HSV has been implicated in cervical neoplasia. Initial evidence came from cytopathological and histological studies (Stern and Longo, 1963; Naib et al., 1966; Naib et al., 1973). Numerous seroepidemiological studies have shown that the frequency and titre of antibodies to HSV types 1 and 2 are higher in sera from individuals with diagnosed cervical anaplasia than in the sera of

controls (Rawls et al., 1969; Nahmias et al., 1970; Skinner et al., 1977). Several viral antigens have been described (Royston and Aurelian, 1970; Aurelian et al., 1973; Strnad and Aurelian, 1976; Dreesman et al., 1980). RNA homologous to HSV DNA in both CIN and CIS have been demonstrated by in situ hybridization studies (McDougall et al., 1980; 1982; Eglin et al., 1981).

Apart from one report (Frenkel et al., 1972), early attempts to demonstrate the presence of HSV DNA in cervical neoplastic tissue failed (zur Hausen et al., 1974; Pagano, 1975). More recently, HSV-2 DNA has been detected by Southern blotting analysis in a small percentage of histologically abnormal cervixes (Park et al., 1983; Manservigi et al., 1986; Prakash et al., 1985; Rotola et al., 1986). Minson (1984) did not, however, detect HSV DNA in 13 epithelial lines cultured from invasive carcinoma in situ tissue.

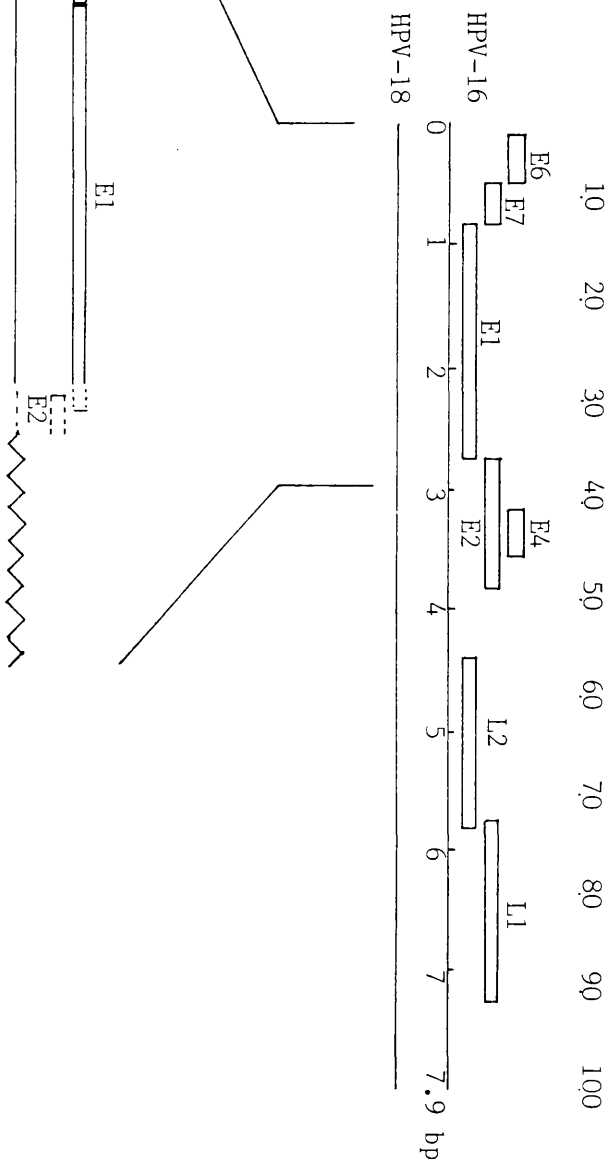
#### Cl.3.2      Oncogenic Potential of HSV

Inactivated HSV-1 and HSV-2 can transform rodent cells in vitro from a normal to a transformed phenotype and the transformed cells are oncogenic in newborn animals (Duff and Rapp, 1971a, 1971b, 1973; Macnab, 1974). Viral RNA, DNA and glycoproteins have been reported to be present in HSV transformed cells (see Galloway and McDougall, 1983 and Macnab, 1987 for reviews). However, no consistent viral product responsible for transformation has been identified. Moreover, viral DNA is not retained in these cells with passage (Frenkel et al., 1976; Galloway and McDougall, 1983; Cameron et al., 1985).

Experiments using defined fragments of viral DNA to transform rodent cells in culture have led to the definition of regions in HSV-1 and HSV-2 responsible for morphological transformation (fig 1.7). In the case of HSV-1, a fragment located between 0.29 and 0.45 map units (mu) is able to transform hamster embryo cells and BALB 3T3 cells (Camacho and Spear, 1978; Reyes et al., 1979). In the case of HSV-2, two

FIG. 1.8

HPV-16 / HPV-18 GENOME STRUCTURE AND CELLULAR INTEGRATION PATTERN

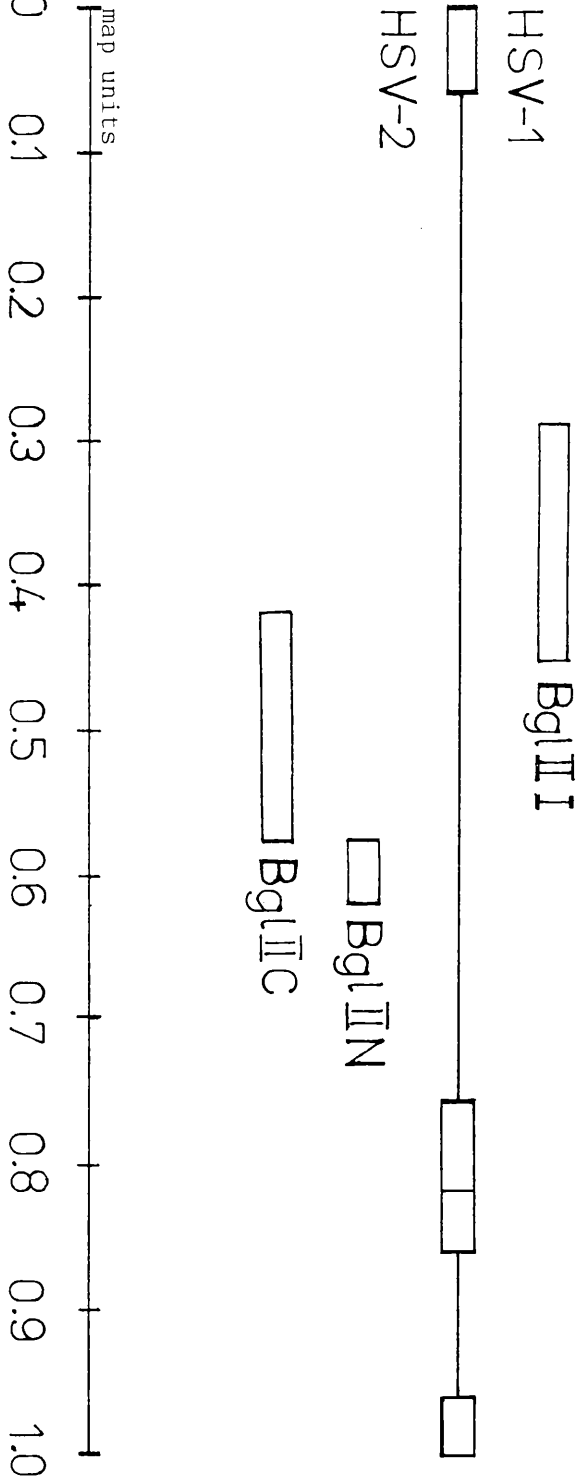


(Adapted from Seedorf et al., 1987 and Schwartz, 1987).

The numbering system is based on analogy with other HPV DNA sequences aligned via the CAAT-box region. Alignment of HPV-16 and HPV-18 sequences is by nucleotide sequence homology. At the top of the figure, the distribution of the ORF's is shown. At the bottom, the integration pattern of HPV-18 DNA into cellular sequences is shown. ORF's are represented as open boxes, HPV DNA sequences as solid lines, cellular sequences as zig-zag lines and viral - cellular junction sites as dotted lines.

FIG. 1.7

TRANSFORMING FRAGMENTS OF HSV-1 AND HSV-2



The location of transforming genes along the HSV genome. The blocks indicate the position of restriction enzyme fragments of HSV-1 (above) and HSV-2 (below) transforming DNA along the prototype arrangement of the HSV genome. (Reproduced from Galloway and McDougall, 1983).

different fragments have been shown to transform rodent cells in culture, depending on the assay used to select transformants. The BglII<sup>n</sup> fragment of HSV-2, which maps between 0.58 and 0.62 mu, has been shown by focus formation in low serum or colony formation in semi-solid medium to alter the growth properties of hamster and rat embryo cells and BALB and NIH 3T3 cells (Reyes et al., 1979; Galloway and McDougall, 1981; Cameron et al., 1985).

A different fragment, BglII<sup>c</sup>, which maps between 0.42 and 0.58 mu, has been shown by conversion to longevity, to cause transformation of Syrian hamster cells (Jariwalla et al., 1980). The BglII<sup>c</sup> fragment can be subdivided into two functional domains. The region from 0.42 to 0.53 mu is responsible for immortalisation of cells (Jariwalla et al., 1983) while the region from 0.53 to 0.58 mu is required for conversion of immortalised cell to a neoplastic phenotype (Jariwalla et al., 1986).

### Cl.3.3      Mechanisms for Transformation by HSV

#### Cl.3.3.1    "Hit and Run" Hypothesis

The failure to detect a consistent set of viral sequences or a specific protein maintained in transformed cells or in human tumours led to the proposal of a "hit and run" mechanism for HSV transformation (Skinner, 1976; Galloway and McDougall, 1983). The identification of sequences of HSV DNA that can transform cells in tissue culture without persisting and without evidence of a transforming protein supports this hypothesis (Galloway et al., 1984; Cameron et al., 1985; Jariwalla et al., 1986). The mechanisms by which HSV may induce morphological transformation have recently been discussed (Macnab, 1987) and will only be briefly summarized here.

#### Cl.3.3.2    Putative Transforming DNA Sequences

A small stem/loop structure in the BglII<sup>n</sup> fragment of HSV-2 (Galloway et al., 1984) and insertion-like sequences in the BglII<sup>c</sup> fragment of HSV-2 (Jones et al., 1986) could be involved in transposition into host DNA where they may activate a cellular oncogene or act as mutagens.

However, such sequences frequently occur in other regions of HSV DNA as well as in randomly shuffled DNA sequences (Shillito et al., in press and quoted by Macnab, 1987). No evidence exists for their biological function.

#### Cl.3.3.3 Ribonucleotide Reductase

HSV-1 and HSV-2 induce novel ribonucleotide reductase activities (Cohen et al., 1972, 1974). There is evidence (Dutia, 1983) that the HSV-1 ribonucleotide reductase is virus encoded although it is not essential for HSV replication in exponentially growing cells in tissue culture (Goldstein and Weller, 1988). Ribonucleotide reductase consists of a large and a small subunit (Bacchetti et al., 1984, 1986; Frame et al., 1985) encoded by two transcripts whose colinear 3' ends map within the BglII<sup>n</sup> fragment of HSV-2 (McLauchlan and Clements, 1983). The small subunit is completely encoded by the BglII<sup>n</sup> fragment. The large subunit is mostly encoded by BglII<sup>c</sup> but the carboxyl terminus lies in BglII<sup>n</sup> (McLauchlan and Clements, 1983), (fig. 1.7).

It has been proposed (Huszar and Bacchetti, 1983) that ribonucleotide reductase could be involved in transformation. The data derived from transformation studies with BglII<sup>n</sup> and BglII<sup>c</sup> (reviewed by Macnab, 1987) show no clear demonstration of the requirement for either the large or the small subunits of ribonucleotide reductase in HSV transformed cells.

#### Cl.3.3.4 Mutagenesis

Both HSV-1 (Schlehofer and zur Hausen, 1982) and HSV-2 (Pilon et al., 1985) have a mutagenic effect on the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene, probably by introducing point mutations (Pilon et al., 1986). Introduction of double-stranded DNA into NIH 3T3 cells mutagenizes the HGPRT gene but the HSV-2 transforming clone, pCB24 is more mutagenic than pBR322 (Brandt et al., 1987) suggesting that mutagenesis may play a role in transformation.

#### C1.3.3.5 Gene Amplification

HSV-1 can cause amplification of genes in cells in a manner similar to chemical carcinogens (Lavi, 1981; Schlehofer et al., 1983; Brandt et al., 1987). Amplification is dependent on the expression of the HSV polymerase gene (Matz et al., 1984; Heilbronn et al., 1985) and is involved with a cellular component being dependent on the cell line used (Matz et al., 1984; 1985; Heilbronn et al., 1985). Brandt et al. (1986) have demonstrated a 2- to 8- fold amplification of HPV-18 DNA by HSV-1 and HSV-2 in HeLa cells, originally derived from malignant tumours of the cervix. In this case, HSV is acting at a late stage in carcinogenesis.

#### C1.3.3.6 Activation of Cellular Genes

The activation of cellular gene expression has already been described (section B1.7.2). The ability of HSV to induce expression of cellular genes and proteins, particularly if they are involved in mediating cell growth, may be an important step in transformation.

### C1.4 ROLE OF HUMAN PAPILLOMAVIRUS IN CERVICAL CANCER

Human papillomavirus (HPV), commonly known as the "wart" virus, is known to affect many sites in the body. There are numerous different types of HPV, at least eight of which (HPV 6, 11, 16, 18, 31, 33, 34 and 35) are specific for the urogenital tract. Development of genital warts is associated with sexual promiscuity (Syrjänen, 1984).

Papillomavirus particles have been demonstrated within koilocytic cells of cervical condylomas by electron microscopy (Meisels et al., 1981). Papillomavirus antigens have also been detected in the same cells showing HPV particles in their nuclei (Gupta et al., 1983).

DNA hybridization studies have shown that HPV types 16 and 18 are strongly associated with high grade CIN and invasive cancer



(Dürst et al., 1983; Boshart et al., 1984; McCance et al., 1985; Prakash et al., 1985; Macnab et al., 1986). However, HPV-16 has also been found in a high percentage of histologically normal tissues (taken from an adjacent site to the tumour tissue), (Macnab et al., 1986) demonstrating that the mere presence of the HPV genome is not sufficient itself to induce a cell to become tumourigenic.

The viral DNA is integrated into the host cell genome in the carcinomas (some of the tumours contain additional, mainly multimeric episomes), whereas it is present as extrachromosomal monomeric episomes in the precursor lesions (Dürst, 1987). In cell lines established from cervical carcinomas e.g. HeLa, C4-1, SW 756, HPV-18 sequences are integrated into the host cell genome by disruption of the papillomavirus genome within the open reading frames (ORF's) of the early (E) genes E1 and E2 (fig. 1.8), thereby joining the 5'-part of the E region (ORF E6-E7-E1 segment) to downstream host cell sequences (Schwartz, 1987). This integration pattern has also been observed in cervical carcinoma biopsies (Dürst, 1987) leading to the speculation that in genital tract lesions, certain alterations in HPV gene expression, resulting from this type of integration event, are required for malignant conversion and/or maintenance of the malignant state. It may also be possible that, in some genital tumours, cis-activation of cellular oncogenes by HPV may contribute to malignant transformation (Dürst et al., 1987).

In HPV-16 and HPV-18 integrated DNA, the E6-E7 ORF's are intact and have the potential to code for proteins. The E7 protein has been detected, using E7-specific antiserum, in cell lines containing HPV-16 or HPV-18 DNA (Smotkin and Wettstein, 1986; Seedorf et al., 1987). In vitro translation of viral enriched (hybrid selected) poly (A)<sup>+</sup> RNA from CaSki cells (contain HPV-16 DNA) and immunoprecipitation has shown the presence of the E6 protein. The transforming potential of the E6 and E7 proteins remains to be verified.

As already mentioned, HPV DNA has been found in a high percentage of

histologically normal tissue (Macnab et al., 1986), suggesting that the presence of the HPV genome is not sufficient to induce transformation. Moreover, cell hybrids, created by fusion of HeLa cells with normal human fibroblast or keratinocyte cells (Stanbridge, 1976; Stanbridge et al., 1982), show tumourigenic properties in vitro (e.g. anchorage independence) but do not form tumours in nude mice, even although the parent HeLa cell line forms tumours in nude mice. It seems therefore, that in vivo, other factors are involved in transformation.

There is evidence that cofactors are required to convert benign lesions caused by HPV to a malignant state. Rabbit papillomas induced by Shope papillomavirus frequently convert into squamous cell carcinomas. This transformation seems to be affected by the genetic constitution of the host and can be accelerated by carcinogens such as tar (Rous and Friedewald, 1944). Bovine papillomas of oesophagus and intestine caused by bovine papillomavirus type 4 (BPV-4) progress to cancer with the aid of bracken as cofactor (Jarrett et al., 1978). In the rare skin lesion, epidermodysplasia verruciformis, papillomas undergo malignant conversion almost exclusively in sites exposed to the sun (Jablonska et al., 1982). Possible cofactors for HPV in cervical cancer include smoking (Trevathan et al., 1983), oral contraceptives (Vessey et al., 1983) and/or HSV (zur Hausen, 1982). It is not known how smoking acts as a cofactor. It may be due to a carcinogenic effect of nicotine or it may reduce the blood supply to tissues. Oral contraceptives contain a mixture of steroids. No one has linked a particular steroid to cervical cancer but steroid metabolism is also recognized to be altered in smokers (Editorial, Lancet, Dec. 1986).

It has been proposed (zur Hausen, 1982) that HSV is responsible for an initiation event and HPV in promotion of cervical carcinogenesis. This would help to reconcile seroepidemiological data linking HSV to genital cancer with the apparent difficulties of detecting HSV DNA in biopsies of genital tumours.

## C1.5      INTERACTION OF HORMONES AND VIRUSES IN CERVICAL CANCER

The association between viruses and cervical cancer and the reports of hormone exposed females being at higher risk of developing cervical cancer has stimulated a limited number of studies on the possible interaction of viruses and hormones in cervical cancer. Possibly HSV-2 acts as an initiator and hormones and/or HPV as promoting agents or perhaps hormones induce changes in cells so that they become more susceptible to viral effects.

Two lines of approach have been taken; in vivo studies on the mouse and in vitro studies on cultured cells. The effect of pregnancy on viral infection in the mouse and human has also been investigated.

### C1.5.1      In Vivo Studies

In an initial study, Muñoz (1973) treated BALB/c mice with various combinations of hormones and HSV-2 and looked at the frequency of precancerous and cancerous lesions of the cervix and vagina which resulted. This study suggested a role for HSV-2 in cervical carcinoma but the role of hormones, if any, was unclear, Nishiura and Nii (1976) examined the effect of oestrogen and progesterone on genital HSV infection in mice and found that progesterone but not oestrogen increased the susceptibility of uterine tissue to virus infection.

There have been reports that genital infection with HSV is 2- to 3-fold more common during pregnancy, a period of high serum progesterone levels (Nahmias et al., 1971) and that pregnancy should be considered as a predisposing factor for primary HSV infection. Studies have shown that the pregnant mouse is more susceptible to viral infections, including HSV-2 (Overall et al., 1975) and that progesterone has a similar effect (Baker and Plotkin, 1978). The reason why progesterone should increase susceptibility to virus infection is not clear but it may be due to an indirect effect through lowering cell mediated immunity (Baker et al., 1980). Alternatively, progesterone may act directly on the vaginal mucosa to allow penetration of HSV-2.

### C1.5.2     In Vitro Studies

Several in vitro studies on the effect of hormones on virus replication and/or transformation have been reported and have shown varying results. Replication of polyoma virus in mouse embryo fibroblasts and mouse 3T3 cells in the presence and absence of various hormones was studied by Morhenn et al. (1973) using plaque assays to measure virus titres. Physiological concentrations ( $10^{-9}\text{M}$  to  $10^{-7}\text{M}$ ) of dexamethasone increased plaque formation at least 10-fold. Cortisol had a similar effect but oestradiol and progesterone had no effect. Costa et al. (1974) carried out a similar investigation with HSV-2 with less striking results. Moreover, different virus yields were obtained according to the virus strain and type of cell used. Tanaka et al. (1984a) found that treatment of human embryonic lung fibroblasts with pharmacological doses of dexamethasone ( $10^{-5}\text{M}$  to  $10^{-8}\text{M}$ ) significantly enhanced human cytomegalovirus (HCMV) replication while oestrogen, progesterone and androgen had no effect. Dexamethasone was also able to overcome the restriction on growth of HCMV in a human hepatoma cell line and in primary epithelial human baby kidney cells (Tanaka et al., 1984b).

Rapp and Turner (1979) looked at the effect of DES on replication and transformation by herpesviruses. They found no significant enhancement of plaque production by HSV-1 or HSV-2 in human embryo fibroblast or in primary rabbit kidney cells treated with DES. However, the frequency of transformation of  $\text{tk}^-$  to  $\text{tk}^+$  mouse cells by HSV was enhanced by pretreatment of cells with DES. Gupta and Rapp (1977) also looked at the influence of steroids on biochemical transformation from  $\text{tk}^-$  to  $\text{tk}^+$ . They found that  $10^{-6}\text{M}$  cortisol and  $10^{-5}\text{M}$  oestradiol inhibited HSV-2 induced transformation and that dexamethasone had very little effect. These experiments using such high concentrations of steroid are not biologically relevant since patients on steroid treatment are subjected to plasma concentrations of hormone of around  $5 \times 10^{-7}\text{M}$ . Thus no clear-cut pattern of the effect of hormones on virus growth has emerged.

## AIM OF PROJECT

The aim of this project was to investigate the possible interaction of steroid hormones and HSV, with particular relevance to the problem of cervical cancer. HPV was not studied as there is no tissue culture system available for growing the virus.

The project was divided into four parts:

- (a) The effect of hormones on virus growth.
- (b) The effect of hormones on virus gene expression.
- (c) The effect of virus infection on steroid receptor levels.
- (d) The effect of virus infection on steroid mRNA levels.

## MATERIALS AND METHODS

## 2.1            MATERIALS

### 2.1.1        Suppliers

Abbott Laboratories, Wokingham, U.K.  
Aldrich Chemical Co., Inc., Gillingham, U.K.  
Amersham International, plc, Amersham, U.K.  
BCL, Lewes, U.K.  
BDH Chemicals Ltd., <sup>C</sup>/o McFarlane Robson Ltd., Glasgow, U.K.  
Bio-Rad Laboratories, Watford, U.K.  
Boehringer Mannheim GmbH, <sup>C</sup>/o BCL, Lewes, U.K.  
BRL-Gibco, Paisley, U.K.  
CAMLAB, Cambridge, U.K.  
Difco Laboratories, West Molesley, U.K.  
Du Pont (U.K.) Ltd., Stevenage, U.K.  
Falcon, <sup>C</sup>/o A & J Beveridge Ltd., Edinburgh, U.K.  
Fluka-Garantie, Glossop, U.K.  
Gibco, Paisley, U.K.  
Koch-Light Laboratories Ltd., Haverhill, U.K.  
Kodak Ltd., Manchester, U.K.  
May & Baker Ltd., Dagenham, U.K.  
Medicell International Ltd., London, U.K.  
Miles Scientific, <sup>C</sup>/o ICN Biomedicals Ltd., High Wycombe, U.K.  
Millipore (U.K.) Ltd., Harrow, U.K.  
NBL Enzymes Ltd., Cramlington, U.K.  
New England Biolabs, <sup>C</sup>/o CP Laboratories, Bishops Stortford, U.K.  
Nunc, <sup>C</sup>/o Gibco, Paisley, U.K.  
Pharmacia Ltd., Milton-Keynes, U.K.  
Polaroid (U.K.) Ltd., St. Albans, U.K.  
Sarstedt Ltd., Leicester, U.K.  
Schleicher & Schuell, <sup>C</sup>/o Anderman & Co. Ltd., Kingston upon Thames, U.K.  
Sigma Chemical Co. Ltd., Poole, U.K.  
E.R. Squibb & Sons Ltd., Hounslow, U.K.  
Sterilin Ltd., Hounslow, U.K.  
Whatman Ltd., <sup>C</sup>/o R & J Wood, Paisley, U.K.

### 2.1.2      Continuous Cell Lines

Baby hamster kidney 21, clone 13 (BHK 21/C13) cells, established by Macpherson and Stoker (1962), maintained in the Institute of Virology. Glasgow, were routinely used to prepare stocks of virus and to titrate virus.

ZR-75-1 cells, derived from the malignant ascitic effusion of a metastatic breast tumour (Engel et al., 1978) were obtained from two sources:

- (a) Imperial Cancer Research Fund (ICRF), Lincoln Inn Fields, London.
- (b) Department of Biochemistry, University of Liverpool.

MCF-7 cells, derived from the pleural effusion of a breast tumour (Soule et al., 1973) were obtained from the same sources as ZR-75-1 cells.

### 2.1.3      Primary Cells

Primary cervical cells were cultured from explants derived from the cervixes of hysterectomy specimens from patients attending the Western Infirmary, Glasgow, with no history of, or colposcopic evidence of CIN or carcinoma.

### 2.1.4      Cell Growth Media

BHK 21 (Glasgow modification of Eagle's medium) was supplied as a 10X concentrate by Gibco and diluted to a final concentration of 1X with sterile distilled water, including supplements (section 2.2.1). Dulbecco's modification of Eagle's medium (DMEM) was supplied as a 1X concentrate by Gibco. Biggar's medium (without phenol red) was supplied as a 2X concentrate by Gibco and diluted with sterile distilled water to a final concentration of 1X.

### 2.1.5      Supplements to Cell Growth Media

Sodium bicarbonate (7.5%), L-Glutamine (200 mM), Penicillin (10 000 U/ml)/



Streptomycin (10 000 µg/ml), Gentamycin (50 mg/ml) and non-essential amino acids were obtained from Gibco.

Mycostatin sterile powder (500 000 U made up in 10 ml distilled water) was obtained from E.R. Squibb & Sons.

Tryptose phosphate broth (10% v/v) was obtained from Difco Laboratories.

Calf serum (CS) was prepared in the Institute of Virology from blood obtained from the ~~Aber~~<sup>at</sup>toire in Dumfries.

Foetal calf serum (FCS) was obtained from Gibco.

Human serum (HS) was prepared in the Institute of Virology from blood obtained from the Blood Transfusion Service, Glasgow, free of any pathogens.

#### 2.1.6      Viruses

The following viruses were grown from stocks provided by Mrs. M. Murphy, Institute of Virology:

- (a) HSV-1, Glasgow strain 17 syn+ (Brown et al., 1973)
- (b) HSV-2, strain HG 52 (Timbury, 1971)
- (c) HSV-1, strain 17 syn+, tsK (Crombie, 1975)

#### 2.1.7      Bacteria

E. coli DH101 bacteria were grown from seed stocks maintained in the Institute of Virology.

#### 2.1.8      Bacteria Culture Media

Bacteria were propagated in L-broth, consisting of 1% (w/v) Bacto-tryptone (Difco Laboratories), 0.5% (w/v) yeast extract (Difco Laboratories), and 0.17M NaCl with the pH adjusted to 7.5.

Agar plates were prepared from L-broth containing 1.5% (w/v) Bacto-agar and sterilized by autoclaving.

These media were supplemented, where appropriate, with ampicillin or tetracycline (100 µg/ml).

L-broth was prepared by the Media Department, Institute of Virology.

### 2.1.9      Fine Chemicals

Chemicals were of 'AnalaR' grade or equivalent and were obtained from the following companies:

|                         |  |
|-------------------------|--|
| Aldrich Chemical Co.    | DATD (N,N'-Diallyltartardiamide)   |
| Amersham International  | ORG 2058   |
| BCL                     | DTT (Dithiothreitol)   |
| Boehringer Mannheim     | Tris [(hydroxymethyl)aminomethane]   |
| Bio-Rad Laboratories    | Ammonium persulphate, TEMED  |
| Difco Laboratories      | Agar (Noble), Bacto-agar, Yeast extract  |
| Fluka-Guarantie         | Formaldehyde, Formamide  |
| Koch-Light Laboratories | Acrylamide, Boric acid, Caesium chloride   |
| Ltd.                    | DMSO (Dimethylsulphoxide), Glycerol, Sodium hydroxide, Sucrose, TCA (Trichloroacetic acid)   |
| May & Baker Ltd.        | Acetic acid glacial, Chloroform, Hydrochloric acid, Methanol   |
| Pharmacia Ltd.          | Dextran T-70, Ficoll, Poly (dA-dT), Bly (dG-dC), Sephadex G-50   |
| Sigma Chemical Co. Ltd. | Acetyl co-enzyme A, Agarose, Bovine serum albumin, Calf thymus DNA, Charcoal (Norit A), Chloramphenicol, Cholera toxin (from <i>Vibrio cholera</i> ), Cycloheximide, Deoxy-ribonucleoside triphosphates, Dextran sulphate, Disulfiram, Epidermal growth factor, Ethidium bromide, Hydrocortisone, Methionine, MOPS (3-[N-Morpholino]propane-sulphonic acid), Oestradiol-17 $\beta$ , PMSF (Phenylmethylsulphonyl fluoride), PVP (Polyvinylpyrrolidone), Progesterone, Salmon sperm DNA, Sodium arsenate, Spermidine, Triton X-100. |

All other chemicals were obtained from BDH Chemicals Ltd.

### 2.1.10      Radiochemicals

The following radiochemicals were obtained from Du Pont (U.K.) Ltd. (NEN Research Products):

|   |                |
|---|----------------|
| [ $\gamma$ - $^{32}\text{P}$ ]-adenosine triphosphates          | 6 000 Ci/m mol |
| [ $\alpha$ - $^{32}\text{P}$ ]-deoxynucleoside triphosphates    | 3 000 Ci/m mol |
| Chloramphenicol, D-Thero-(Dichloroacetyl-1,2- $^{14}\text{C}$ ) | 60 Ci/m mol    |

The following radiochemicals were obtained from Amersham International:-

|  |                |
|--|----------------|
| [ $^3\text{H}$ ]-Oestradiol-17 $\beta$ | 89 Ci/m mol    |
| [ $^3\text{H}$ ]-ORG 2058              | 51 Ci/m mol    |
| [ $^{35}\text{S}$ ]-methionine         | > 800 Ci/m mol |

#### 2.1.11 Stains and Dyes

Bromophenol blue (BFB) and Giemsa (2-[2-(4-hydroxyphenyl)-2-benzimidazolyl]-6-[1-methyl-4-piperazyl]benzimidazole trihydrochloride) were obtained from BDH Chemicals Ltd.

Hoescht 33258 and xylene cyanole were obtained from Sigma Chemical Co.Ltd. Protein dye reagent concentrate was obtained from Bio-Rad Laboratories.

#### 2.1.12 Enzymes

|                                 |                               |
|---------------------------------|-------------------------------|
| Deoxyribonuclease               | Boehringer Mannheim           |
| DNA polymerase $\bar{\text{I}}$ | BRL-Gibco                     |
| Lysozyme                        | Sigma Chemical Co. Ltd.       |
| Proteinase K                    | Boehringer Mannheim           |
| Restriction endonucleases       | BRL-Gibco or NBL Enzymes Ltd. |
| Ribonuclease type A             | Sigma Chemical Co. Ltd.       |
| T4 polynucleotide kinase        | BRL-Gibco                     |
| Trypsin                         | Difco Laboratories            |

#### 2.1.13 Photographic Material

|  |                      |
|--|----------------------|
| Amfix  | May & Baker Ltd.     |
| Du Pont Cronex Lighting Plus<br>intensifying screens | Du Pont (U.K.) Ltd.  |
| Dx developer and X-omat S film                       | Kodak Ltd.           |
| 667 film   | Polaroid (U.K.) Ltd. |

#### 2.1.14      Miscellaneous Material

|   |                             |
|---|-----------------------------|
| ER EIA kit  | Abbott Laboratories         |
| Oligolabelling kit  | Pharmacia Ltd.              |
| Rabbit reticulocyte lysate  | Sigma Chemical Co. Ltd.     |
| RNA mol. wt. markers  | BRL-Gibco                   |
| Ecoscint  | University Chemistry stores |
| EN <sup>3</sup> HANCE   | Du Pont (U.K.) Ltd.         |
| Chromotography paper  | Whatman Ltd.                |
| Hybond-N membrane   | Amersham International      |
| Nitrocellulose membrane   | Schleicher & Schuell        |
| Thin-layer chromatography paper<br>(0.25 mm silica pre-coated plastic sheets) | CAMLAB                      |
| Petri dishes, 35 mm   | Nunc                        |
| Petri dishes, 50 mm and 90 mm   | Sterilin                    |
| Plastic tissue culture flasks,<br>80 cm <sup>2</sup> and 175 cm <sup>2</sup>  | Nunc                        |
| Plastic tissue culture roller bottles,<br>850 cm <sup>2</sup>                 | Falcon                      |
| Eppendorf reaction vials  | S <del>t</del> arstedt Ltd. |
| Millex filters, 0.22 µm and 0.45 µm   | Millipore (U.K.) Ltd.       |
| Plastic centrifuge tubes, 15 ml and 50 ml                                     | Falcon                      |
| Visking dialysis membrane   | Medicell International      |

#### 2.1.15      Standard Solutions

##### 2.1.15.1      Buffers

|                 |  |
|-----------------|--|
| Chamber         | 0.05M Tris, 0.05M Glycine, 0.1% (w/v) SDS                                |
| 100X Denhardt's | 0.02% (w/v) Ficoll, 0.02% (w/v) PVP,<br>0.02% (w/v) BSA                  |
| 2X HBS          | 0.28M NaCl, 1.5mM Na <sub>2</sub> HPO <sub>4</sub> , 0.05M Hepes, pH 7.2 |

|                    |   |   |
|--------------------|---|---|
| HE                 |   | 20 mM Hepes, 1.5 mM EDTA, pH 7.4  |
| HED                |   | 20 mM Hepes, 1.5 mM EDTA, 0.25 mM DTT, pH 7.4   |
| 10X Kinase         |   | 1M Tris-HCl, 1M MgCl <sub>2</sub> , 1M DTT, 0.1M Spermidine, 0.25M EDTA, pH 7.5   |
| 10X MOPS           |   | 0.2M MOPS, 0.05M Na acetate, 0.01M EDTA, pH 7.0   |
| Nuclear Extraction | A | 0.01M Hepes, 1.5mM MgCl <sub>2</sub> , 0.01M KCl, 0.5mM DTT, 0.01M Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , 0.5 mM PMSF, pH 7.9  |
|                    | B | 0.02M Hepes, 25% (v/v) Glycerol, 0.42M NaCl, 1.5 mM MgCl <sub>2</sub> , 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.01M Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , pH 7.9                    |
|                    | C | 0.02M Hepes, 0.02M KCl, 1 mM MgCl <sub>2</sub> , 2 mM DTT, 17% (v/v) Glycerol, pH 7.9   |
| 10X NTB            |   | 0.05M Tris-HCl, 5 mM MgCl <sub>2</sub> , 1 mM DTT, pH 7.8   |
| PBS                |   | 0.17M NaCl, 3.4 mM KCl, 1 mM Na <sub>2</sub> HPO <sub>4</sub> , 2 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.2   |
| 'React'            |   | obtained as a 10X concentrate from BRL-Gibco  |
| RNA Extraction     | A | 0.15M NaCl, 0.01M Tris-HCl, 1.5 mM MgCl <sub>2</sub> , 0.65% NP 40, pH 7.8  |
|                    | B | 7.0M Urea, 0.35M NaCl, 0.01M EDTA, 0.01M Tris-HCl, 1% (w/v) SDS, pH 7.8   |
| Separating Gel     |   | 1.5M Tris-HCl, 0.4% (w/v) SDS, pH 8.9   |
| Sodium Phosphate   |   | 1M NaH <sub>2</sub> PO <sub>4</sub> , 1M Na <sub>2</sub> HPO <sub>4</sub> , pH 7.0  |
| Stacking Gel       |   | 0.5M Tris-HCl, 0.4% (w/v) SDS, pH 6.7   |
| Sucrose-Tris       |   | 25% (w/v) Sucrose in 0.05M Tris-HCl, pH 8.0   |
| 20X SSC            |   | 3M NaCl, 0.3M tri-Sodium Citrate, pH 7.4  |
| 20X SSPE           |   | 3.6M NaCl, 0.2M Na <sub>2</sub> H <sub>2</sub> PO <sub>4</sub> , 0.02M EDTA, pH 7.4   |
| TAE                |   | 0.04M Tris-acetate, 1 mM EDTA, pH 7.8   |
| TBE                |   | 0.089M Tris-borate, 0.089M Boric acid, 2mM EDTA   |
| TE                 |   | 0.01M Tris-HCl, 1 mM EDTA, pH 7.5 or 8.0  |
| TEN                |   | 0.04M Tris-HCl, 0.01M EDTA, 0.12M NaCl, pH 7.8  |
| Tris-Saline        |   | 0.14M NaCl, 0.03M KCl, 0.28 mM Na <sub>2</sub> HPO <sub>4</sub> , 1 mg/ml dextrose, 0.25 mM Tris, 0.005% phenol red, pH 7.4, supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin |
| Triton Lytic Mix   |   | 2 % (v/v) Triton X-100, 0.06M EDTA, 0.05M Tris-HCl, pH 8.0  |

### 2.1.15.2 Dyes/Stains

|               |  |
|---------------|--|
| BFB dye       | 10X TBE buffer, 10% (w/v) Ficoll, 0.05% (w/v) bromophenol blue   |
| BFB/XC dye    | 10% (w/v) Ficoll, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanole in distilled water.                           |
| Giemsa stain  | 1.5% (w/v) suspension of Giemsa in glycerol, heated at 56°C for 1.5 – 2 h and diluted with an equal volume of methanol.  |
| Hoescht stain | Stock solution of Hoescht 33258, 100 µg/ml (w/v) in distilled water, stored at 4°C in the dark for a maximum of 1 month. |

### 2.1.15.3 Steroid and Growth Factor Solutions

|  |  |
|--|--|
| Cholera toxin  | <u>Stock:</u> 1.2 ml sterile, distilled water was added to a 1 mg vial of cholera toxin to give a $10^{-5}$ M solution and stored at 4°C.<br><u>Dilutions:</u> 50 µl of $10^{-5}$ M stock solution was diluted in Hepes-buffered Earle's salts + 0.1% BSA to give a $10^{-8}$ M solution which was filter sterilized and stored in 2 ml aliquots at -20°C. |
| Epidermal Growth Factor (EGF)                        | 5 ml sterile, distilled water was added to a 100 µg vial of EGF. A 1:10 dilution was made in sterile Hepes-buffered Earle's salts + 0.1% BSA and stored in 2 ml aliquots at -20°C.   |
| Hydrocortisone                                       | 20 µg hydrocortisone was dissolved in 10 ml distilled water + 10 ml methanol to give a 1 µg/ml stock which was stored at -20°C.  |
| Oestradiol-17β, Progesterone and Diethylstilboestrol | were made up as $10^{-3}$ M stock solutions in ethanol and stored at -20°C.  |

2.2.1 Culture of Continuous Cell Lines

BHK 21/C13 cells were cultured in BHK 21 medium supplemented by 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25% (<sup>v</sup>/v) sodium bicarbonate, 10% (<sup>v</sup>/v) tryptose phosphate broth, 5 or 10% (<sup>v</sup>/v) calf serum (CS).

Stocks of cells were grown routinely in 80 oz glass bottles (washed and autoclaved in the Institute of Virology), seeded at a density of  $4 \times 10^7$  cells in 200 ml medium containing 10% CS. 50 mm petri dishes were seeded at a density of  $2 \times 10^6$  cells per dish in 4 ml medium containing 5% CS.

ZR-75-1 and MCF-7 cells were grown in DMEM supplemented by 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 or 10 % foetal calf serum (FCS).

Stocks of cells were grown routinely in 175 cm<sup>2</sup> plastic tissue culture flasks seeded at a density of  $4 \times 10^6$  cells per flask in 50 ml DMEM + 10% FCS. For large scale experiments, 850 cm<sup>2</sup> plastic tissue culture roller bottles were seeded at a density of  $2 \times 10^7$  cells per bottle in 100 ml DMEM + 10% FCS. Most experiments were done in 80 cm<sup>2</sup> tissue culture flasks, seeded at a density of  $2 \times 10^6$  cells per flask in 25 ml DMEM + 10% FCS.

All cells were harvested by washing once with versene (6 mM EDTA dissolved in PBS containing 0.0015% phenol red) and twice with trypsin-versene (one volume of 25% (<sup>w</sup>/v) Difco trypsin dissolved in Tris-saline, plus four volumes of versene), incubating at 37°C for 5 min and resuspending at the required concentration in the appropriate medium. The cells were seeded as described above and grown in an atmosphere of 5% CO<sub>2</sub> at 37°C.

### 2.2.2 Test for Mycoplasma Infection

Cells were grown on glass coverslips for 48h, washed in PBS, then fixed in methanol:acetone (3:1), cooled to -20°C for 4 - 5 min. After air drying, the cells were stained with Hoescht stain (10 µl of stain diluted to 0.05 µg/ml) for 10 min at ambient temperature. The coverslips were then washed in three changes of PBS and mounted in 20% glycerol in PBS on glass slides. The cells were examined under a UV-fluorescent microscope for evidence of mycoplasma infection.

### 2.2.3 Primary Culture of Cervical Cells

Cervical cells were cultured from explants derived from hysterectomy specimens of premenopausal patients with no evidence of CIN or carcinoma. The cervix was excised from the uterus and vagina and washed three times in PBS to remove red blood cells. The ectocervix was removed by cutting 2 mm below the transformation zone (fig.1.6). Explants were prepared by carefully removing muscle and stroma using a sharp, sterile scalpel until a thin epithelium of cells remained. The epithelium was finely minced with sterile, curved scissors. The tissue was kept moist in PBS.

10 - 20 small explants were seeded per 35 mm plastic petri dish and just covered with a small volume of FCS. The explants were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in FCS for 4 - 18 h to allow them to attach to the surface of the dish. 2 ml of cervical medium (DMEM supplemented by 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 µg/ml hydrocortisone, 10<sup>-10</sup>M cholera toxin (to inhibit growth of fibroblast cells), 10 µg/ml gentamycin, 100 U/ml mycostatin and 10% FCS) was added to the dishes and incubation continued for a further 2 - 3 days. Any floating explants were removed and fresh cervical medium containing 10 ng/ml epidermal growth factor added. The medium was changed twice weekly. Cells formed an almost confluent monolayer in 2 - 3 weeks.



#### 2.2.4 Preparation of Frozen Cell Stocks

Frozen stocks of ZR-75-1 and MCF-7 cells were prepared as follows: Cells were grown in 175 cm<sup>2</sup> flasks in DMEM + 10% FCS until they were approximately 80% confluent. Cells were harvested using trypsin-versene (section 2.2.1), pelleted by centrifugation at 1 000 rpm for 5 min at 4°C and resuspended in 6 ml DMEM + 10% FCS + 10% DMSO. 2 ml aliquots of cell suspension were transferred to small, glass vials. Cells were frozen in two stages; an overnight incubation at -70°C followed by long-term storage at -170°C under liquid nitrogen.

#### 2.2.5 Recovery of Frozen Cells

Cells recovered from the -170°C freezer were thawed rapidly at 37°C and transferred to an 80 cm<sup>2</sup> flask containing 10 ml pre-warmed medium (DMEM + 10% FCS + 1% non-essential amino acids). Cells were incubated for 24h at 37°C in an atmosphere of 5% CO<sub>2</sub>. The medium was then replaced with fresh medium to remove any dead cells and to dilute out the DMSO used in the storage medium. Cells were grown as before (2.2.1).

#### 2.2.6 Preparation of Heat-Inactivated, Charcoal-Stripped Serum

500 ml FCS was heat-inactivated at 56°C for 30 min. 1.25 g Norit A charcoal was mixed with 0.125 g dextran T-70 in 125 ml PBS and stirred gently for 18h at 4°C. The dextran-coated charcoal was pelleted by centrifugation at 10 000 rpm for 20 min at 4°C (GSA rotor), added to 500 ml heat-inactivated FCS and stirred gently for 18h at 4°C. The charcoal was pelleted by centrifugation as before.

The stripped serum passed through a series of filters including Whatman No. 1 paper, 0.45 µm and 0.22 µm Millex Millipore filters and finally through a 0.22 µm sterile filter. The sterile, stripped medium was aliquoted into 10 ml amounts in glass bottles and stored at -20°C.

### 2.2.7      Virus Infection

The medium covering cells to be infected was discarded and the appropriate amount of virus added in as small a volume of PBS or medium as was necessary to cover the cells. HSV-1 and HSV-2 were adsorbed for 1h at 37°C. The mutant virus, tsK, was adsorbed at 38.5°C for 1h. Cells were drained of medium containing unabsorbed virus and given fresh medium. Cells infected with wild-type virus were incubated at 37°C. Cells infected with tsK were incubated at 38.5°C.

### 2.2.8      Production of Virus Stocks

#### 2.2.8.1    HSV-2

HSV-2 stocks were prepared by infecting just subconfluent BHK 21/C13 cells in 80 oz glass bottles at a m.o.i. of 0.003 pfu/cell. Virus was adsorbed for 1h at 37°C and the cells overlaid with 25 ml BHK 21 medium containing 5% CS. Infected cells were incubated at 37°C for 2 days or 31°C for 3 days. Cells were harvested by shaking into the medium which was collected and spun in 50 ml sterile, glass centrifuge bottles for 15 min at 4°C. The majority of the supernatant was removed, leaving a small residue sufficient to resuspend the infected cell pellet. The infected cell suspension was transferred to a sterile, glass, conical flask and sonicated for 15 min at 4°C in a Cole-Palmer ultrasonic cleaning bath. Cells were pelleted by centrifuging at 1000 rpm for 15 min at 4°C. The supernatant was removed and stored at -70°C. The cell pellet was resuspended in the minimum amount of medium and sonicated again for 15 min at 4°C to release any remaining cell-associated virus. The cells were then pelleted as before. The second supernatant was removed and stored at -70°C.

#### 2.2.8.2    HSV-1

HSV-1 stocks were prepared by infecting just subconfluent BHK 21/C13 cells in 80 oz glass bottles at a m.o.i. of 0.002 pfu/cell. Virus was

adsorbed, grown and harvested and centrifuged at 1 000 rpm for 15 min at 4°C, as described for HSV-2 (2.2.8.1). HSV-1 is released into the growth medium therefore the supernatant containing HSV-1 was centrifuged at 12 000 rpm for 3h at 4°C or at 35 000 rpm for 45 min at 4°C to pellet the virus. The pellet was resuspended in a minimum amount of growth medium, sonicated and pelleted twice as for HSV-2 (2.2.8.1). To harvest any cell-associated HSV-1, the original pellet was resuspended in growth medium, sonicated and pelleted once as already described (2.2.8.1). All virus preparations were stored at -70°C.

### 2.2.8.3     tsK

Preparation of tsK stocks was similar to preparation of HSV-1 stocks (2.2.8.2) except that adsorption and growth of virus was at 31°C, the permissive temperature for tsK infection.

## 2.2.9        Titration of Virus

### 2.2.9.1     HSV-1 and HSV-2

Serial 10-fold dilutions of virus were made in PBS + 2% CS. 100 µl inocula was added to almost confluent monolayers of BHK 21/C13 cells in 50 mm petri dishes. Virus was adsorbed for 1h at 37°C, then 4 ml BHK 21 medium containing 2% HS added. Infected cells were incubated for a further 2 days at 37°C. The medium was discarded into flasks containing chloros. Monolayers were stained with Giemsa for 30 min at ambient temperature. Excess Giemsa stain was removed by washing in water. Plates were dried and the number of plaques counted under a dissecting microscope. Titres were expressed as the number of plaque-forming units per cell (pfu/cell).

### 2.2.9.2     tsK

Titration of tsK was identical to titration of HSV-1 and HSV-2 except that adsorption and growth of virus was at 31°C. Infected cells were incubated for 3 days at 31°C before staining with Giemsa.

#### 2.2.10      Preparation of Bacteria Competent for Transformation with DNA

An isolated colony of E. coli DH101 bacteria was picked from an agar plate and grown in 10 ml L-broth overnight at 37°C with shaking. 100 ml L-broth was inoculated with 2 ml of the overnight bacterial culture and grown with vigorous shaking at 37°C to an optical density of 0.2 at 630 nm (3 - 4 h). The culture was chilled on ice for 10 min, then centrifuged at 6 000 rpm for 10 min at 4°C. The pellet was drained, resuspended in 50 ml of 10 mM ice-cold, sterile CaCl<sub>2</sub> and incubated on ice for 20 min. The bacteria were centrifuged as before, resuspended in 50 ml of 75 mM CaCl<sub>2</sub> and incubated on ice for 20 min. The cells were centrifuged again as before and resuspended in 3 ml of 75 mM CaCl<sub>2</sub>. Competent bacteria were stored at -70°C.

#### 2.2.11      Transformation of Bacteria with DNA

0.1 - 1.0 µg plasmid DNA in a volume of 1 µl was added to 100 - 150 µl competent bacteria (2.2.10), gently mixed and incubated on ice for 40 min. The bacteria were then incubated at 37°C for 3 min and plated directly onto media selective for an appropriate plasmid resistance gene. Plates were incubated at 37°C overnight to allow colonies of transformed bacteria to grow. Plates were stored at 4°C.

#### 2.2.12      Preparation of Plasmid DNA

An overnight culture of bacteria transformed with the appropriate plasmid was prepared from seed stocks and used to inoculate 1 litre of L-broth supplemented by 100 µg/ml ampicillin (or tetracycline where appropriate). The bacteria were grown at 37°C with vigorous shaking to an optical density of 0.8 at 630 nm. Chloramphenicol was then added to a final concentration of 100 µg/ml to stop the bacteria from multiplying while still allowing replication of plasmid DNA. Cultures were incubated for a further 16 - 24 h at 37°C with vigorous shaking.

The bacteria were pelleted by centrifugation at 8 000 rpm for 15 min

at 4°C in a Sorvall RC-5B refrigerated superspeed centrifuge using a GS3 rotor and resuspended in 8 ml ice-cold sucrose-tris. The bacterial suspension was distributed between two 50 ml SS34 centrifuge tubes on ice. To each tube was added 2 ml of 5 mg/ml lysozyme for 5 min at 4°C followed by 3 ml of 250 mM EDTA, pH 8.0 for 5 min at 4°C and finally by 9 ml Triton lytic mix for 20 min at 4°C. Cell debris was removed by centrifugation at 15 000 rpm for 60 min at 4°C in a Sorvall superspeed centrifuge using an SS34 rotor. The lysate was incubated with 200 µg/ml proteinase K + 0.1% SDS at 37°C for 1h, then extracted once with phenol (saturated with TE buffer, pH 8.0) and chloroform. The nucleic acids were precipitated by incubation with a half volume of isopropanol and 0.2M sodium acetate for 30 min at ambient temperature. The precipitate was pelleted by centrifugation at 10 000 rpm for 30 min (Sorvall centrifuge, rotor, SS34), then resuspended in TE buffer, pH 8.0.

The plasmid DNA was purified by centrifugation to equilibrium in CsCl/EtBr gradients. Gradients were prepared to give a final density of 1.55 g CsCl per ml and 500 µg/ml EtBr in a final volume of 20 ml. Centrifugation was in a Sorvall OTD 50 ultra-centrifuge using a TV865B rotor at 40 000 rpm for a minimum of 16h at 15°C. The DNA bands (upper = host DNA; lower = plasmid DNA) were visualized by long-wave UV-light and the plasmid DNA collected by tube puncture. EtBr and CsCl were removed by extracting three times with isopropanol saturated with CsCl followed by extensive dialysis against TE buffer, pH 8.0 at 4°C. The DNA was precipitated with isopropanol in the presence of 0.2M sodium acetate, resuspended in distilled water and quantitated spectrophotometrically ( $\lambda = 260$  nm). DNA was stored either at 4°C for immediate use or at -20°C for longer storage.

#### 2.2.13 Quantitation of Nucleic Acids by Optical Density (OD) Measurement at 260 nm.

The absorption at 260 nm of various dilutions of DNA or RNA samples was measured using quartz microcuvettes in a Perkin Elmer double beam R4 spectrophotometer. The concentration was estimated assuming

that OD = 1.0 is equivalent to 50 µg/ml double-stranded DNA, 40 µg/ml RNA and 20 µg/ml oligonucleotide DNA.

#### 2.2.14 Quantitation of Nucleic Acid by Agarose Gel Electrophoresis

Small samples (0.25 - 1.0 µg) of DNA were electrophoresed with known standard concentrations of λ DNA on 0.6% (<sup>w</sup>/v) agarose gels in TBE buffer containing 0.5 µg/ml EtBr. The DNA was visualized by UV-illumination of the gels and the concentration of the unknown determined by visual comparison of the fluorescence with that of the standard λ DNA. Gels were photographed under short-wave UV-light using Polaroid type 667 film.

#### 2.2.15 Transfection of Cultured Cells with Plasmid DNA

All solutions used in transfection of cells were filter sterilized through a 0.22 µm Millipore filter before use. Pre-packed, sterile plastic tubes were used for the reaction. The quantities given below are for cells grown in 80 cm<sup>2</sup> flasks.

20 µg plasmid DNA in 420 µl TE buffer was added to tube 1 followed by 60 µl 2M CaCl<sub>2</sub> added dropwise with mixing. The contents of tube 1 were then transferred dropwise with mixing to tube 2 containing 480 µl of 2X HBS, pH 7.2. Incubation was for 30 min at ambient temperature to allow formation of a DNA-CaPO<sub>4</sub> precipitate. This mixture was then added dropwise onto the cells.

Cells were incubated at 37°C for 24h, the medium discarded and replaced with fresh, pre-warmed medium. Incubation was continued for a further 24h. The medium was then discarded, the cell monolayer washed twice with PBS and the cells harvested by incubation in TEN buffer, pH 7.8 for 5 min followed by cell scraping. Cells were pelleted by centrifugation at 1000 rpm for 5 min at 4°C and resuspended in 300 µl of 250 mM Tris, pH 7.8 on ice. Disruption of cells was by three cycles of freeze/thawing. Cell debris was pelleted by centrifugation at 3 000

rpm for 5 min. Aliquots of cell cytosol were used for protein estimation (2.2.14) and CAT assay (2.2.15) and the rest stored at  $-20^{\circ}\text{C}$ .

#### 2.2.16 Estimation of Protein Concentration by Bio-Rad Assay

The Bio-Rad protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

Bio-Rad protein dye reagent was supplied as a 5-fold concentrate. For the standard assay procedure (2.2.16.1), the dye reagent was diluted 5-fold in distilled water and filtered through Whatman No. 1 paper. The diluted dye could be stored for up to two weeks in a glass container at ambient temperature.

##### 2.2.16.1 Standard Assay Procedure (for measuring 20 - 140 $\mu\text{g}$ protein)

A stock solution of 1 mg/ml BSA was made up in distilled water for use in protein standards. The stock BSA solution was stored at  $-20^{\circ}\text{C}$ . From the 1 mg/ml BSA stock solution, the following aliquots were transferred to plastic tubes - 0, 100, 200, 300, 400, 500 and 600  $\mu\text{l}$  - and the volume made up to 1 ml in each case with distilled water. These solutions were used as "standard curve" solutions of 0 - 600  $\mu\text{g}/\text{ml}$  BSA.

100  $\mu\text{l}$  of each standard solution and 100  $\mu\text{l}$  of each appropriately diluted sample were placed in clean, dry test tubes. 5.0 ml diluted dye reagent was added and the mixture vortexed gently. After a period of between 5 min and 60 min, the optical density at 595 nm was measured. A graph of  $\text{OD}_{595}$  versus the protein concentration of the standards was drawn. The protein concentrations of the unknown samples were read from the graph.

#### 2.2.16.2 Microassay Procedure (to measure 1 - 20 µg protein)

BSA standards ranging from 1 to 25 µg/ml were made up in distilled water. 800 µl standards and appropriately diluted samples were placed in clean, dry test tubes. 200 µl concentrated dye reagent was added and the mixture vortexed gently. After a period of between 5 min and 60 min, the optical density at 595 nm was measured. A graph of OD<sub>595</sub> versus the protein concentrations of the samples were read from the graph.

#### 2.2.17 Chloramphenicol Acetyl Transferase (CAT) Assay

Standard "CAT mix" consisting of 14 µl H<sub>2</sub>O, 1 µl acetyl CoA, 0.5 µl [<sup>14</sup>C]-chloramphenicol per assay was made up as a larger stock solution (1 ml) and stored at 4°C. 15.5 µl CAT mix was aliquoted into eppendorf tubes and incubated at 37°C for 5 min before addition of each 25 µl cell extract. Incubation at 37°C was continued for 30 min.

The reaction was stopped by the addition of 200 µl ethyl acetate with vigorous mixing and transfer of the tubes to ice. Organic and aqueous layers were separated by centrifugation in an eppendorf microfuge at high speed for 1.5 min. The top, organic layer was removed, transferred to a new eppendorf tube and dried down by lyophilization. The chloramphenicol products were resuspended in 25 µl ethylacetate and spotted onto silica thin-layer chromatography (TLC) plates. Ascending chromatography was in 95% chloroform and 5% methanol.

The TLC plates were autoradiographed overnight. The position of the spots was marked, cut out and the amount of radioactivity counted with "Ecoscint" on an Intertechnique SL 4000 liquid scintillation counter. The percentage of acetylated : non-acetylated chloramphenicol products was calculated. The results were expressed as n moles acetylated chloramphenicol/mg protein/h.



### 2.2.18      Restriction Enzyme Digestion

DNA was incubated at 37°C for at least 4h with the appropriate restriction enzyme (approximately 10 units of enzyme per µg DNA) in the presence of the appropriate "React" buffer in a total volume (made up with distilled water) not less than 10X the volume of the restriction enzyme added. For digestion with two enzymes, the reactions were carried out simultaneously if the enzymes operated under the same salt conditions. If not, then the two digestions were done separately. After the first digestion, the DNA was extracted once with phenol and chloroform and precipitated at -70°C with two volumes of ethanol in the presence of 0.2M sodium acetate. The DNA was resuspended in a small volume of distilled water and the appropriate enzyme and "React" buffer added. Incubation was at 37°C for at least 4h as before. Restriction enzyme digestion was checked on agarose mini-gels (2.2.19).

### 2.2.19      Agarose Mini-Gel Electrophoresis

Agarose gel concentrations of 0.6% (<sup>w</sup>/v) were used routinely in mini-gels to visualize and quantitate plasmid DNA. 1% agarose mini-gels were used to check the integrity of RNA samples and to check the result of restriction enzyme digestion of DNA.

The agarose was dissolved in TBE buffer by boiling, cooled to approximately 50°C and 0.5 µg/ml EtBr added before pouring. Samples were prepared in TBE buffer and 0.1X volume of BFB dye. Electrophoresis was carried out at ambient temperature at about 60V for 30 min in TBE buffer containing 0.5 µg/ml EtBr. Gels were photographed under short-wave UV-irradiation using Polaroid type 667 film.

Cloned OR3 cDNA (fig. 6.1, section 6.2.2) was excised from its vector sequences by digestion with Eco R1. The resulting fragments were

Cloned OR3 cDNA (fig. 6.1, section 6.2.2) was excised from its vector sequences by digestion with Eco R1. The resulting fragments were

separated on a 1% agarose slab gel (prepared in the same way as minigels, 2.2.19). Electrophoresis was carried out at 30V for 18h. The lower band was cut from the gel with a sharp, sterile scalpel under long-wave UV-light. The excised band was placed inside dialysis tubing along with TBE buffer. The DNA was electroeluted in TBE buffer at 100V. To release any DNA concentrated on the dialysis membrane, the current was reversed for a few seconds. The buffer inside the dialysis tubing containing the eluted DNA was transferred to a 50 ml Falcon centrifuge tube. The DNA was extracted once with phenol and once with chloroform, then precipitated twice with two volumes of ethanol in the presence of 0.2M sodium acetate at -20°C overnight. The DNA was pelleted and resuspended in distilled water and quantitated spectrophotometrically ( $\lambda = 260 \text{ nm}$ ).

#### 2.2.21 Purification of DNA from Low Melting Point Agarose

Cloned actin cDNA ( fig. 6.9, section 6.5.3) was excised from its vector sequences by digestion with Xba I. The resulting fragments were separated on a 1% low-melting point agarose slab gel.

The agarose was dissolved in TAE buffer by boiling, cooled to approximately 50°C and 0.5  $\mu\text{g/ml}$  EtBr added. The gel was poured and set at ambient temperature. The sample was prepared in TAE buffer and 0.1X volume BFB dye added. Electrophoresis was carried out at ambient temperature in TAE buffer containing 0.5  $\mu\text{g/ml}$  EtBr at 30V for 18h.

The separated bands were visualized under long-wave UV-light and the lower, actin band excised with a sharp, sterile scalpel. The gel slice was cut into smaller pieces and transferred to an eppendorf tube. The agarose was melted at 70°C, the volume measured and the concentration of actin DNA estimated by calculating the fraction of the total plasmid DNA which was actin DNA. The agarose containing actin DNA was stored in 50  $\mu\text{l}$  aliquots in eppendorf tubes at -20°C.

### 2.2.22      Nondenaturing Polyacrylamide Gel Electrophoresis

This method was used for gel retardation assays (2.2.31)

A stock solution of 44% (<sup>w</sup>/v) acrylamide : 0.8% bisacrylamide (NN'-methylene-bisacrylamide) was prepared in distilled water and filtered through "Amberlite" monobed resin MB-1 and Whatman No. 1 filter paper.

50 ml of an 8% polyacrylamide gel mix was prepared as follows:

|                          |            |
|--------------------------|------------|
| Stock acrylamide         | 9.0 ml     |
| 20X TBE                  | 2.5 ml     |
| H <sub>2</sub> O         | 38.5 ml    |
| 25% ammonium persulphate | 0.2 ml     |
| TEMED                    | 15 $\mu$ l |

Ammonium persulphate and TEMED were added last and the gel poured immediately. Electrophoresis was carried out in TBE buffer at 30 mA for 2h. The gel was then soaked in a solution of 10% acetic acid for 20 min and dried under vacuum at 80°C for 2h.

### 2.2.23      Denaturing Polyacrylamide Gel Electrophoresis

This method was used to purify synthetic oligonucleotides after synthesis. A stock solution of 50% acrylamide (20:1 acrylamide : bisacrylamide) was made up in distilled water and filtered through "Amberlite" monobed resin MB-1 and Whatman No. 1 filter paper. For purification of the ER oligonucleotides (section 6.2.1), a 15% acrylamide gel mix containing 7M urea was made up in TBE buffer. 400  $\mu$ l ammonium persulphate and 40  $\mu$ l TEMED were added to 75 ml gel mix and the gel poured immediately.

The oligonucleotide, prepared as described in section 2.2.25, was loaded and electrophoresis carried out in TBE buffer at 30 mA for 3 - 4 h. The oligonucleotide band was visualized as a shadow cast under long-wave UV-light, cut out and eluted as described in section 2.2.25.

## 2.2.24 SDS Polyacrylamide Gel Electrophoresis

This method was used to separate denatured proteins on the basis of mol. wt.

A stock solution of 30% acrylamide : 0.75% bisacrylamide was made up in distilled water and filtered through "Amberlite" monobed resin MB-1 and Whatman No. 1 filter paper. Standard separating, stacking and chamber buffers used for these gels are described under section 2.1.15.1.

7% acrylamide, single concentration gels were prepared as follows:

### Separating Gel

|                            |            |
|----------------------------|------------|
| Acrylamide stock           | 12.0 ml    |
| Gel Buffer (pH 8.9)        | 12.0 ml    |
| H <sub>2</sub> O           | 24.0 ml    |
| ammonium persulphate (10%) | 0.3 ml     |
| TEMED                      | 40 $\mu$ l |

### Stacking Gel

|                                 |            |
|---------------------------------|------------|
| Acrylamide stock (+ 0.78% DATD) | 1.0 ml     |
| Stacking Gel Buffer (pH 6.7)    | 1.5 ml     |
| H <sub>2</sub> O                | 3.5 ml     |
| ammonium persulphate (10%)      | 50 $\mu$ l |
| TEMED                           | 15 $\mu$ l |

The separating gel was poured and allowed to set with a layer of butan-1-ol on the top to ensure a level surface. Once the separating gel had set, the butanol was removed and the surface of the gel washed with  $\frac{1}{4}$  strength stacking gel buffer. The stacking gel was then poured on top of the separating gel and allowed to set.

Protein samples were prepared in a final concentration of 1X "boiling mix" with 0.1X volume of BPB dye and denatured by boiling for 3 min.

A 3X concentrate of boiling mix was prepared as follows:

Stacking Gel Buffer (1.0 ml), 20% SDS (1.0 ml), Glycerol (1.0 ml) and  $\beta$ -mercaptoethanol (0.5  $\mu$ l).

Electrophoresis was for 3h at 40 mA in chamber buffer. The gel was fixed in 50% methanol : 7% acetic acid for 1h, washed in 5% methanol : 7% acetic acid for 1h then in H<sub>2</sub>O for 30 min. The gel was then soaked in "En<sup>3</sup>hance" for 30 min, washed for 2 x 15 min in H<sub>2</sub>O and dried under vacuum at 80°C for 2h. The gel was autoradiographed for various lengths of time.

#### 2.2.25      Synthesis and Purification of Synthetic Oligonucleotides

Oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer by Dr. J. McLauchlan, Institute of Virology, Glasgow.

The newly synthesized oligonucleotide (approximately 200 µg) was incubated at 55°C for 5h, then split into four aliquots in eppendorf tubes and dried down by lyophilization. Three of the tubes were stored at -70°C until required.

The contents of the fourth tube were resuspended in 50 µl water plus 50 µl sample buffer (0.28 ml 10X TBE, 1.7 ml H<sub>2</sub>O, 8 ml deionized formamide). The mixture was vortexed, heated at 90°C for 3 min and immediately chilled on ice. 0.1X volume of BFB/XC dye was added to the sample before loading onto a denaturing polyacrylamide gel (2.2.23).

After electrophoresis, the oligonucleotide band was cut out (2.2.23), ground into small pieces in a 5 ml plastic tube and covered with 2 ml ammonium acetate elution buffer (see below). The oligonucleotide was eluted by shaking in this buffer for 18h at 37°C. The solution was then filtered through glass wool to remove the acrylamide. The oligonucleotide was precipitated with 2.5 volumes of ethanol at -20°C for 18h, pelleted by centrifugation at high speed in an eppendorf microfuge and resuspended in 50 µl distilled water. The DNA was quantitated spectrophotometrically (2.2.13). The oligonucleotide was stored at -20°C.

### Ammonium Acetate Elution Buffer

|                     |            |
|---------------------|------------|
| 3M ammonium acetate | 1.0 ml     |
| 0.25M EDTA (pH 7.8) | 24 $\mu$ l |
| 20% SDS             | 30 $\mu$ l |
| H <sub>2</sub> O    | 5.0 ml     |

## 2.2.26 In Vitro Labelling and Purification of cDNA Probes

### 2.2.26.1 Nick-Translation

The following ingredients were added to an eppendorf tube on ice:-

|                             |  |
|-----------------------------|--|
| 5 $\mu$ l                   | 0.25 $\mu$ g DNA                           |
| 5 $\mu$ l                   | 1% BSA                                     |
| 5 $\mu$ l                   | 10X NTB                                    |
| 2 $\mu$ l                   | 2 mM dCTP                                  |
| 2 $\mu$ l                   | 2 mM dGTP                                  |
| 5 $\mu$ l                   | [ <sup>32</sup> P]-dATP                    |
| 5 $\mu$ l                   | [ <sup>32</sup> P]-dTTP                    |
| 17 $\mu$ l                  | H <sub>2</sub> O                           |
| 2 $\mu$ l                   | DNA polymerase <u>I</u> (2 units/ $\mu$ l) |
| 2 $\mu$ l                   | DNase (10 <sup>-5</sup> M)                 |
| <u>50 <math>\mu</math>l</u> |  |

The reaction mix was incubated at 37°C for 2 min to start the reaction, then at 15°C for approximately 60 min. The reaction was stopped by transferring the tube to ice.

### 2.2.26.2 Random Priming or Oligolabelling

This method was used to label actin cDNA purified on low melting point agarose (2.2.21).

The agarose containing actin cDNA was melted at 70°C and 50 ng was transferred to an eppendorf tube. The total volume was made up to 31  $\mu$ l

with distilled water. The actin cDNA was denatured by heating at 90°C for 10 min, then transferred to a 37°C water bath for 5 min. At ambient temperature, the following ingredients were added:-

|              |                                    |
|--------------|------------------------------------|
| 31 µl        | denatured DNA                      |
| 10 µl        | reagent mix *                      |
| 2 µl         | BSA                                |
| 5 µl         | [ $\alpha$ - <sup>32</sup> P]-dCTP |
| 2 µl         | Klenow                             |
| <u>50 µl</u> |                                    |

\*Reagent mix, BSA and Klenow fragment of DNA polymerase I were supplied in the Pharmacia oligolabelling kit. The reagent mix contained dATP, dGTP, dTTP, p(dN)<sub>6</sub> and buffer.

The reaction mix was incubated at ambient temperature for 2 - 4 h. The reaction was terminated by the addition of 20 µl "stop" buffer (supplied in kit) and 180 µl H<sub>2</sub>O.

#### 2.2.26.3 Isotope Incorporation and Specific Activity of cDNA Probes

Duplicate Whatman No. 1 filter paper discs were spotted with 1 µl of the nick-translation or oligolabelling reaction mixtures. One disc was transferred directly to a scintillation vial and 2 ml Ecoscint added. The other disc was given 3 x 5 min washes in 5% ice-cold TCA to precipitate the DNA and remove unincorporated nucleotide triphosphates. The disc was then rinsed in ethanol, dried under a lamp, transferred to a scintillation vial and 2 ml Ecoscint was added. The washed and unwashed discs were counted on an Intertechnique SL 4000 liquid scintillation counter. The percentage isotope incorporation was calculated by the percentage (bound counts/total counts). The specific activity of the probe was calculated as cpm/µg DNA.

#### 2.2.26.4 Purification of cDNA Probes

A 1 ml sterile, plastic syringe was plugged with glass wool and filled with a suspension of Sephadex G-50 equilibrated in TE buffer. The syringe was then suspended in a 15 ml Falcon centrifuge tube containing a 1.5 ml eppendorf tube and centrifuged at 1 000 rpm for 1.5 min. The packed Sephadex volume was at least 1.8 ml. The eppendorf tube containing "flow-through" buffer was replaced with a fresh tube. The volume of the nick-translation mix was made up to 250  $\mu$ l with 200  $\mu$ l distilled water. The volume of the oligolabelling mix was already 250  $\mu$ l due to the addition of "stop" buffer and water. The sample was gently layered on top of the packed Sephadex column, then centrifuged at 1 000 rpm for 1.5 min. Unincorporated triphosphates remained on the column while radiolabelled DNA passed through the column into the eppendorf below. Recovery of DNA was 85%.

#### 2.2.26.5 Denaturation of cDNA Probes

Probes were denatured before use in hybridization reactions either by  
(a) boiling for 5 min followed by rapid cooling on ice or  
(b) incubation with NaOH at a final concentration of 0.2M for 10 min at ambient temperature. HCl was then added to a final concentration of 0.2M to neutralize the NaOH.

#### 2.2.27 In Vitro Labelling and Denaturation of Oligonucleotide Probes

##### 2.2.27.1 Kinase Reaction

Oligonucleotide DNA was end-labelled with  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  using T4 polynucleotide kinase. The following reaction mixture was incubated at 37°C for 1h, then transferred to ice to stop the reaction.

Isotope incorporation and the specific activity of the probe were determined as described in section 2.2.26.3.



### Reaction Mixture:

|                             |   |
|-----------------------------|---|
| 2 $\mu$ l                   | (0.2 $\mu$ g) oligonucleotide DNA       |
| 3 $\mu$ l                   | 10X kinase buffer                       |
| 2 $\mu$ l                   | (10 units) T4 polynucleotide kinase     |
| 5 $\mu$ l                   | $[\gamma\text{-}^3\text{P}]\text{-ATP}$ |
| 18 $\mu$ l                  | H <sub>2</sub> O                        |
| <u>30 <math>\mu</math>l</u> |   |

### 2.2.27.2 Denaturation of Oligonucleotide Probes

The oligonucleotide reaction mixture was made up to a total volume of 200  $\mu$ l by the addition of 170  $\mu$ l formamide. This mixture was heated at 90°C for 3 min, cooled rapidly on ice and added directly to the hybridization mixture. The presence of unincorporated  $[\gamma\text{-}^3\text{P}]\text{-ATP}$  did not affect the hybridization reaction.

### 2.2.28 Preparation of Cellular RNA

#### 2.2.28.1 Preparation of Crude Cell Extracts

Cells grown in 50 mm petri dishes (and treated with E<sub>1</sub> or infected with virus) were washed twice in ice-cold PBS, harvested by scraping with a rubber policeman into 1 ml PBS and transferred to an eppendorf tube. Cells were pelleted by centrifugation for 30 sec at high speed in an eppendorf microfuge, The cells were resuspended in 45  $\mu$ l TE buffer and 5  $\mu$ l of 5% (v/v in TE buffer) NP 40, mixed and incubated on ice for 5 min. A further 5  $\mu$ l of 5% NP 40 was added, the samples mixed and centrifuged at high speed in an eppendorf microfuge for 2.5 min. 50  $\mu$ l supernatant was transferred to a sterile eppendorf tube containing 30  $\mu$ l of 20X SSC + 20  $\mu$ l formaldehyde, mixed and incubated at 60°C for 15 min. Samples were stored at -70°C.

#### 2.2.28.2 Extraction of Cytoplasmic Cellular RNA

Cell growth medium was removed and the monolayers washed twice in ice-cold PBS. Cells were harvested by scraping with a rubber policeman and pelleted by centrifugation at 1 000 rpm for 5 min. Cells were resuspended and incubated in 250 µl RNA extraction buffer A for 3 min on ice (quantities are for a confluent 80 cm<sup>2</sup> flask of cells). Lysed cells were centrifuged at 3 000 rpm for 5 min to pellet cell debris and nuclei. The cytosol supernatant was removed and added to an equal volume of RNA extraction buffer B in an eppendorf tube. RNA was extracted three times with an equal volume of phenol/chloroform (1:1) and finally with an equal volume of chloroform alone. RNA was precipitated in two volumes of ethanol at -20°C overnight and pelleted by centrifugation in an eppendorf microfuge at high speed for 10 min. The pellet was resuspended in sterile, distilled water and quantitated spectrophotometrically (2.2.13). The integrity of the RNA was checked by mini-gel agarose electrophoresis (2.2.19). RNA was stored at -70°C.

#### 2.2.29 In Vitro Translation of RNA

Rabbit reticulocyte lysate was obtained from Sigma Chemical Co. Ltd. and stored in 100 µl aliquots at -70°C.

12 µl lysate and 1.5 µl [<sup>35</sup>S]-methionine were mixed together and warmed to 30°C. 1 µg total, cytoplasmic RNA was added in a volume of 1.5 µl sterile, distilled water. The mixture was vortexed and incubated at 30°C for 1h. 15 µl of 0.1M EDTA, 1% (w/v) methionine, 100 µg/ml RNase were added and the mixture incubated for a further 15 min at 30°C to terminate the reaction. Samples were stored at -20°C or -70°C until a convenient time for running an SDS polyacrylamide gel (2.2.24).

## 2.2.30 Slot-Blot Analysis of RNA

### 2.2.30.1 Transfer of RNA to Nitrocellulose Membrane

Various dilutions of RNA samples were made in 50% (V/v) formamide, 6% (V/v) formaldehyde, 10X SSC in a total volume of 100  $\mu$ l. The samples were denatured at 65°C for 5 min, cooled rapidly to 4°C and transferred to nitrocellulose membrane via a Schleicher & Schuell slot-blot apparatus and water pressure. The membrane was pre-wetted in 10X SSC. After transfer of samples, the membrane was air-dried and baked under vacuum for 2h at 80°C.

### 2.2.30.2 Hybridization with ER Oligonucleotide Probes

Nitrocellulose slot-blots were prehybridized for 3h at 42°C in 50% (V/v) formamide, 3X SSC, 1X Denhardt's solution, 150  $\mu$ g/ml denatured salmon sperm DNA in a total volume of 10 ml. Hybridization with ER oligonucleotide probes (-1 or -2) was in 50% formamide and 5X SSC at 42°C for 18 - 24 h.

Blots were washed for 1h at 42°C in prehybridization solution followed by 1h at ambient temperature in 3X SSC. Blots were exposed to X-ray film ("flashed" and "unflashed") with Du Pont intensifying screens at -70°C for 1 - 3 days.

### 2.2.30.3 Hybridization with the OR3 cDNA Probe

The following conditions were outlined by Prof. Chambon when he sent us the OR3 cDNA plasmid:

Nitrocellulose membrane was prehybridized in 50% (V/v) formamide, 5X SSC, 5X Denhardt's solution (with no BSA), 50 mM sodium phosphate buffer (pH 7.0), 0.1% SDS, 0.5 mg/ml denatured salmon sperm DNA, for 4 - 6 h at 42°C.

Hybridization with denatured, nick-translated OR3 cDNA was in 50% (v/v) formamide, 5X SSC, 1X Denhardt's solution (with no BSA), 20 mM sodium phosphate buffer (pH 7.0), 0.1% SDS, 10% (w/v) dextran sulphate and 0.5 mg/ml denatured salmon sperm DNA for 18 - 24 h at 42°C.

Blots were washed as follows:

- (a) 2X SSC, 1% SDS for 2 x 10 min at ambient temperature
- (b) 1X SSC, 0.1% SDS for 30 min at 52°C

Blots were autoradiographed and if a more stringent wash was required then blots were washed in:

- (c) 0.1X SSC, 0.1% SDS for 30 min at 52°C

## 2.2.31 Northern Blot Analysis of RNA

### 2.2.31.1 Size Fractionation of RNA by Denaturing Agarose Gel Electrophoresis

25 µg RNA was denatured in 50% (v/v) formamide, 6% (v/v) formaldehyde and 1X MOPS buffer in a total volume of 60 µl for 5 min at 65°C, then cooled rapidly to 4°C. BFB/XC dye was added (0.1X volume) and the samples loaded onto a denaturing 1.2% (w/v) agarose slab gel containing 6% (v/v) formaldehyde and 1X MOPS buffer.

Ethidium bromide stainable markers, obtained from BRL, were used as mol. wt. size markers. 6 µg standard RNA (containing 1 µg of each RNA mol. wt. marker) was denatured in a similar way to the sample RNA and loaded on the same gel.

Electrophoresis was carried out in 1X MOPS buffer at 80 - 100 V for 4h. The marker track was cut off from the rest of the gel and stained overnight in 0.5 µg/ml ethidium bromide. The track was destained in distilled water for 1h and then photographed under short-wave UV-light. The position of the RNA markers was also marked on paper of the same size as the gel so that a direct comparison could be made with the position of hybridized bands on Hybond membrane to which the sample RNA was transferred.

#### 2.2.31.2 RNA Transfer to Hybond-N Membrane

The gel was transferred to a sheet of Whatman 3 mm paper on a glass plate supported in a tray of 20X SSC. A piece of Hybond-N membrane, cut to the exact size of the gel, was laid directly on top of the gel. Six pieces of Whatman 3 mm paper, cut 2 mm smaller than the gel size, were laid on top of the membrane. A weighted stack of dry paper towels, cut to the same size as the filter paper, was placed on top. The blot was left for 16 - 24 h at ambient temperature to allow complete transfer of the RNA to the membrane. Thereafter, the membrane was removed, air-dried, wrapped in cling-film and placed, RNA-side down, on a UV short-wave transilluminator for 5 min to crosslink the RNA to the membrane.

#### 2.2.31.3 Hybridization

The blot was prehybridized in 50% (v/v) formamide, 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS and 0.1 mg/ml denatured salmon sperm DNA for 1 - 4 h at 42°C.

Hybridization with the appropriate radiolabelled, denatured probe was carried out in 50% (v/v) formamide, 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS, 10% (w/v) dextran sulphate, 0.1 mg/ml denatured salmon sperm DNA for 16 - 24 h at 42°C.

#### 2.2.31.4 Washing of Blots Probed with Actin Radiolabelled cDNA

- (a) 2X SSPE, 0.1% SDS for 30 min at 65°C
- (b) 1X SSPE, 0.1% SDS for 2 x 30 min at 65°C
- (c) 0.1X SSPE, 0.1% SDS for 15 min at 65°C

Washing steps (a) and (b) resulted in a single, specific band corresponding to the 2 kb actin mRNA. This band remained after the very stringent wash (c).

#### 2.2.31.5 Washing of Blots Probed with Radiolabelled ER Oligonucleotide

- (a) 2X SSPE, 0.1% SDS for 30 min at ambient temperature
- (b) 0.1X SSPE, 0.1% SDS for 2 x 30 min at ambient temperature

These washing conditions resulted in a single, specific band corresponding to the 6.2 kb ER mRNA.

#### 2.2.31.6 Reprobing of Blots

The first probe was removed by washing the blot for 1 - 2 h at 65°C in 0.005M Tris-HCl, 0.002M EDTA, 0.1X Denhardt's solution, pH 8.0. Removal of the probe was checked by autoradiography. Blots were prehybridized and hybridized as before.

#### 2.2.31.7 Storage of Blots

The damp membrane was wrapped in cling-film and silver foil on a glass plate and stored at 4°C in the dark for short-term storage (up to 1 month) and at -20°C or -70°C for long-term storage.

## 2.2.32      Extraction of Nuclear Protein

Cells were grown in plastic roller bottles. The cells were harvested after the appropriate treatment with hormones or virus infection, by washing twice in ice-cold PBS and scraping the cells into 25 ml PBS. Cells were pelleted by centrifugation at 2 500 rpm for 10 min at 4°C. Cells were resuspended in five packed cell volumes of ice-cold PBS and pelleted again. All subsequent steps were carried out at 4°C.

Cells were resuspended in five packed cell volumes of nuclear extraction buffer A (2.1.14), incubated for 10 min at 4°C and pelleted as before. Cells were resuspended in two packed cell volumes of buffer A and lysed by ten strokes of a Dounce B type pestle (checked microscopically). Nuclei and cell debris were pelleted by centrifuging at 2 500 rpm for 10 min at 4°C and the supernatant discarded. The pellet was resuspended in two packed cell volumes of buffer A and centrifuged for 20 min at 14 500 rpm at 4°C. The supernatant was discarded, the nuclear pellet resuspended in nuclear extraction buffer B (2.1.14), (3 ml/10<sup>9</sup> cells) and dounced with ten strokes of a type B pestle. The nuclear suspension was incubated in buffer B for 30 min at 4°C with gentle stirring. The nuclei were pelleted by centrifugation at 14 500 rpm for 20 min at 4°C. The supernatant containing nuclear protein was collected. Ammonium sulphate (0.33 g/ml) was added with gentle stirring on ice for 15 min. The nuclear proteins were pelleted by centrifugation at 14 500 rpm for 20 min at 4°C, then redissolved in nuclear extraction buffer C (2.1.<sup>15.1</sup>~~14~~), (1/12 th volume of supernatant). The protein solution was dialysed for 6h at 4°C against two changes of 100 volumes of buffer C. The insoluble proteins were removed by centrifugation at 14 500 rpm for 20 min at 4°C. The protein content of the supernatant was assayed by the Bio-Rad assay (2.2.16). The supernatant of soluble nuclear protein was aliquoted into 100 µl amounts, "snap-frozen" in dry ice/ ethanol and stored at -70°C.

### 2.2.33 Gel Retardation Assay

This assay was used to test for DNA-binding proteins which interact with specific DNA sequences. The principle of the assay is that both DNA and DNA bound to protein can enter native polyacrylamide gels but that DNA/protein complexes migrate at a much slower rate than free DNA. Provided the DNA is radiolabelled, protein/DNA complexes and free DNA can be visualized by autoradiography.

The conditions described below for looking at proteins which recognize the HSV IE activator sequence TAATGARAT (where R = purine) were optimized by Dr. R. Thompson and Dr. A. Bailey in the Institute of Virology, who also supplied the materials for the DNA-binding reaction. Preparation of protein extracts is described in section 2.2.30. The sequence of the TAATGARAT oligonucleotide probe is given in section 4.5.2.

#### 2.2.33.1 Protein/DNA Binding Reaction

The following components were mixed together and incubated for 20 min at ambient temperature:-

|  |                             |
|--|-----------------------------|
| 5X Binding Buffer*                               | 5 $\mu$ l                   |
| [ $\gamma$ - $^{32}$ P]-labelled TAATGARAT probe | 0.2 ng, 10 cps              |
| poly (dA-dT):poly(dG-dC), (1:1) competitor       | 2 $\mu$ g                   |
| protein extract                                  | 0 - 30 $\mu$ g              |
| H <sub>2</sub> O                                 |                             |
| <u>Final Volume</u>                              | <u>25 <math>\mu</math>l</u> |

|                                |                     |
|--------------------------------|---------------------|
| * <u>Binding Buffer (5X)</u> : | 125 mM Hepes        |
|                                | 5 mM EDTA           |
|                                | 25 mM DTT           |
|                                | 250 mM NaCl         |
|                                | 50 % Glycerol (v/v) |

All dilutions of protein extracts were in 1X Binding Buffer.



#### 2.2.33.2 Fractionation of the Products of the Protein/DNA Binding Reaction and Autoradiography

BFB/XC dye (0.1X volume) was added to the protein/DNA binding reaction mixture before electrophoresis through an 8% nondenaturing polyacrylamide gel as described in section 2.2.22.

Once the gel was dried, it was autoradiographed overnight at  $-70^{\circ}\text{C}$ .

#### 2.2.34 [ $^3\text{H}$ ]-Steroid Solutions for ER and PR Assays

##### 2.2.34.1 Preparation of [ $^3\text{H}$ ]- $\text{E}_2$ Solutions

Stock [ $^3\text{H}$ ]- $\text{E}_2$  was prepared from the Amersham supply at  $5 \times 10^{-7}\text{M}$  and stored in ethanol at  $-20^{\circ}\text{C}$ . A stock solution of unlabelled DES at  $1.25 \times 10^{-4}\text{M}$  was made up in ethanol and stored at  $-20^{\circ}\text{C}$ .

Two radiolabelled assay solutions were prepared as follows:

- (a) 24  $\mu\text{l}$  DES was aliquoted into a glass bottle and the ethanol evaporated under a gentle stream of  $\text{N}_2$ .  
24  $\mu\text{l}$  [ $^3\text{H}$ ]- $\text{E}_2$  was then added and the volume of ethanol made up to 30  $\mu\text{l}$ .  
970  $\mu\text{l}$  HED buffer was added, making the total volume 1 ml.
- (b) 24  $\mu\text{l}$  [ $^3\text{H}$ ]- $\text{E}_2$  was aliquoted into a glass bottle and the volume of ethanol made up to 30  $\mu\text{l}$ .  
970  $\mu\text{l}$  HED buffer was added making the total volume 1 ml.

The final concentration of [ $^3\text{H}$ ]- $\text{E}_2$  in the exchange assay was  $30 \times 10^{-10}\text{M}$  and DES was present in 250-fold excess.

##### 2.2.34.2 Preparation of [ $^3\text{H}$ ]-ORG 2058 Solutions

Stock [ $^3\text{H}$ ]-ORG 2058 was prepared from the Amersham supply at  $5 \times 10^{-7}\text{M}$  and stored in ethanol at  $-20^{\circ}\text{C}$ . Unlabelled ORG 2058 was made up at a concentration of  $5 \times 10^{-5}\text{M}$  and stored at  $-20^{\circ}\text{C}$ .

Two radiolabelled assay solutions were made up as follows:

- (a) 40  $\mu$ l unlabelled ORG 2058 was aliquoted into a small glass bottle and the ethanol evaporated under a gentle stream of  $N_2$ . 40  $\mu$ l of labelled ORG 2058 was then added followed by 960  $\mu$ l HED buffer containing 10% glycerol.
- (b) 40  $\mu$ l labelled ORG 2058 was aliquoted into a small glass bottle. 960  $\mu$ l HED buffer containing 10% glycerol was added.

Final concentration of [ $^3H$ ]-ORG 2058 in the exchange assay was  $50 \times 10^{-10}M$  and unlabelled ORG 2058 was in 100-fold excess.

#### 2.2.35 Whole Cell [ $^3H$ ]-Steroid Exchange Assay for ER and PR

Growth medium was discarded and the cell monolayers washed twice in ice-cold PBS. Cells were scraped into PBS and pelleted by centrifugation at 1 000 rpm for 5 min at 4°C. The cells were resuspended in HED buffer containing 1.5 mM  $MgCl_2$  and 10% glycerol, pH 7.4.

150  $\mu$ l cell suspension was incubated with 50  $\mu$ l (radiolabelled ligand  $\pm$  excess unlabelled ligand), (2.2.34.1 and 2.2.34.2), for 2h at ambient temperature. 100  $\mu$ l aliquots from each tube were then added to 5 ml aliquots of 0.9% (w/v) NaCl immediately prior to pouring onto a pre-wetted Whatman GF/C filter disc (2.5 cm) held in a Millipore filter apparatus. The tube which had contained the saline was washed out with 5 ml saline, and this was poured onto the filter also. The chimney of the apparatus was washed with 3 x 4 ml aliquots of saline, then removed and the very edge of the filter washed with 3 ml saline. The filters were placed in scintillation vials and 4 ml Ecoscint added. Samples were counted in a LKB liquid scintillation counter with a counting efficiency of 35%.

### 2.2.36 Cytosol Assay for ER - Dextran-Coated Charcoal Method

Growth medium was discarded and the cell monolayers washed twice in ice-cold PBS. The cells were scraped into PBS from the plastic tissue culture flask and pelleted by centrifugation at 1 000 rpm for 5 min at 4°C. The cells were resuspended in HED buffer, pH 7.4 and sonicated for 3 - 5 min at 4°C in a Cole-Palmer ultrasonic cleaning bath (fixed, gentle setting). Cell debris was pelleted by centrifugation at 2 500 rpm for 5 min at 4°C. The supernatant was collected and tested for ER by [<sup>3</sup>H]-E<sub>2</sub> exchange assay using dextran-coated charcoal to remove unbound steroid.

150 µl cell cytosol was incubated with 50 µl ( [<sup>3</sup>H]-E<sub>2</sub> ± 250-fold excess DES ), (2.2.34.1), for 18h at 4°C. 200 µl dextran-coated charcoal solution (0.5% (w/v) Norit A charcoal, 0.005% (w/v) dextran T-70 in 10% (v/v) glycerol, 1.5 mM EDTA, 20 mM Hepes, pH 7.4) was added to each tube and the tubes mixed. Charcoal treatment was continued on ice for 15 min with periodic mixing. At the end of this time, the charcoal was pelleted by centrifugation at 2 000 rpm for 5 min at 4°C. Aliquots of 200 µl from each supernatant were transferred to scintillation vials, 4 ml of Ecoscint added and the vials counted in an LKB scintillation counter with a counting efficiency of 35%.

### 2.2.37 Cytosol Assay for ER - Enzyme Immunoassay Method

Cell cytosol was prepared as described in 2.2.36. Measurement of ER by the enzyme immunoassay (EIA) was exactly as described in the manual accompanying the Abbott EIA kit. The basis of the assay is described in section 5.2.<sup>4</sup>~~23~~.

## RESULTS

### 3. EFFECT OF STEROID HORMONES ON REPLICATION OF HSV-2 IN VITRO

#### 3.1 INTRODUCTION

A limited number of studies have been done on the effect of steroid hormones on virus replication in cultured cells (section C1.5.2). The results varied with the virus involved, the cell type used and the hormone given to the cells.

This section describes the replication of HSV-2 in primary cultures of human cervical cells and in the human breast cancer cell line, ZR-75-1 in the presence and absence of the steroid hormones, oestradiol ( $E_2$ ) and progesterone (Pg).

#### 3.2 GROWTH AND CHARACTERIZATION OF ECTOCERVICAL CELLS

Primary ectocervical cells were cultured from explants of normal cervical tissue as described in section 2.2.3.

Fig. 3.1 shows ectocervical cells which have grown out from an explant of normal cervical tissue. The cells are epithelial as evidenced by the presence of desmosomes and tonofilaments (fig. 3.2).

#### 3.3 INFECTION OF ECTOCERVICAL CELLS WITH HSV-2

##### 3.3.1 Method

The cells were grown in cervical growth medium (section 2.2.3) for 2 - 3 weeks. The medium was changed to DMEM + 2% HIDCC-FCS for 2 days to reduce endogenous steroid hormone levels to a minimum.

The appropriate hormone was given to the cells 24h prior to virus infection and was present throughout the course of infection. As it was difficult to obtain confluent monolayers of cells, the number of cells per plate was estimated using a standard grid method previously developed by Dr. S. Walkenshaw and Dr. J. Macnab. Cells were infected

FIG. 3.1      GROWTH OF PRIMARY ECTOCERVICAL CELLS

Two photographs of a 10 day old culture of ectocervical cells.  
Magnification X80.

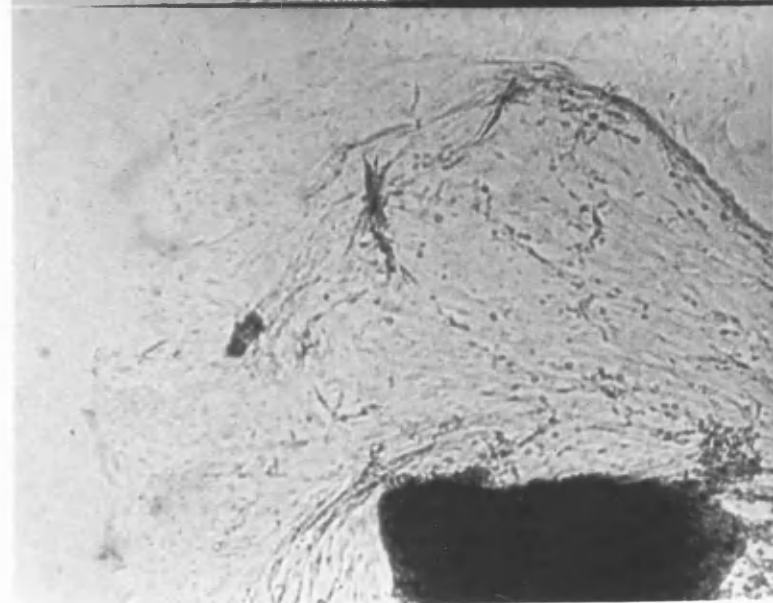
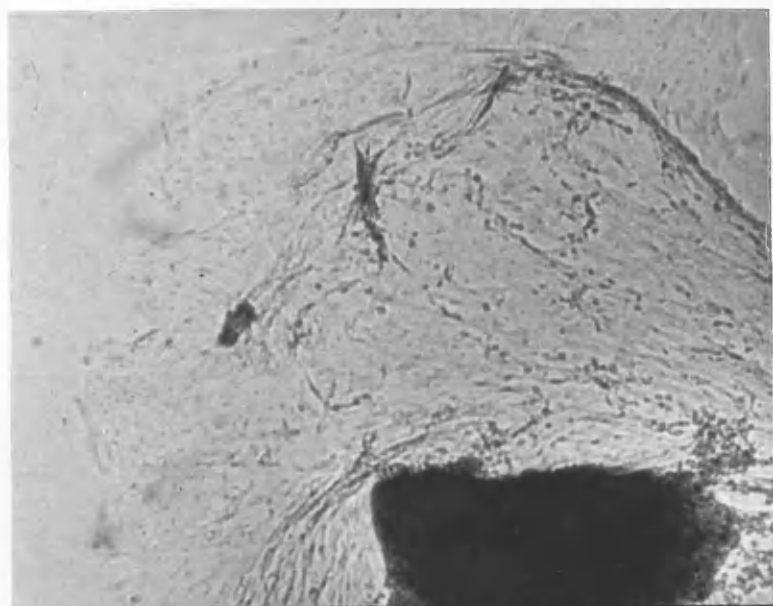


FIG. 3.2      ELECTRON MICROSCOPY OF ECTOCERVICAL CELLS

Electron micrograph of ectocervical cells showing the presence of desmosomes (D) and tonofilaments (T).

Magnification    X45 000

The electron microscopy was kindly carried out by Jim Aitken (Institute of Virology).





with HSV-2 at a m.o.i. of 1 pfu/cell for 48h resulting in extensive c.p.e. (fig. 3.3).

Cells were harvested by scraping into the cell medium. Virus was extracted by sonicating the cells in a Cole-Palmer ultrasonic cleaning bath for 5 min at 4°C. Cells were pelleted by centrifugation at 1 000 rpm for 5 min. The supernatant was stored at -70°C until a suitable time for titration. Virus was titrated as described in section 2.2.9.

### 3.3.2 Results

Table 3.1 shows the virus yields from cells treated with hormones compared to untreated cells. In all cases, productive infection yielded a burst size of 10 pfu/cell. Neither physiological ( $10^{-9}\text{M}$ ,  $10^{-8}\text{M}$ ) nor pharmacological ( $10^{-7}\text{M}$ ,  $10^{-6}\text{M}$ ) doses of  $\text{E}_2$  or Pg (tables 3.1A and 3.1B) significantly altered the virus titre. The combination of  $\text{E}_2$  and Pg together did not alter the virus titre (table 3.1C). Thus, under these conditions, neither  $\text{E}_2$  nor Pg seemed to have any effect on the growth of HSV-2 in ectocervical cells.

### 3.3.3 Problems Involved in Culturing Primary Cervical Cells

A number of difficulties were encountered when culturing primary cervical cells. Only a limited number of suitable hysterectomy samples were available. These were collected from the theatre and taken to the Pathology Department of the Western Infirmary. The pathologists examined the tissue (under non-sterile conditions) for any sign of abnormal tissue and cut off a small piece of normal cervical tissue which was transferred to a sterile container with sterile PBS on ice and taken back to the laboratory. A maximum of a dozen 35 mm petri dishes of explants were seeded from each piece of cervix. A separate batch of cells was used for each experiment although within experiments, each treatment was performed in triplicate. Cells were not able to be passaged. Explants from some cervix samples grew better than others.

TABLE 3.1

Effect of E<sub>2</sub> and/or Pg on the Replication of HSV-2 in  
Primary Cervical Cells

Primary ectocervical cells were grown and maintained in 35 mm petri dishes as described in section 3.3.1 prior to the start of the experiment. The number of cells per dish was estimated by a standard grid method. Triplicate dishes of cells were treated  $\pm$  a range of concentrations (see table) of:

- A. oestradiol-17 $\beta$  (E<sub>2</sub>)
- B. progesterone (Pg)
- C. E<sub>2</sub> + Pg (10<sup>-9</sup>M only)

for 24h prior to infection with HSV-2. Cells were infected with HSV-2 at low multiplicity (1 pfu/cell) for 48h in the continued presence of the same hormones. Cells were harvested, virus extracted and titrated as described in section 3.3.1. The results are expressed as total pfu/cell  $\pm$  standard deviation (S.D.) and divided by the control value (i.e. HSV-2 alone) to give relative titre values.

TABLE 3.1

EFFECT OF E<sub>2</sub> AND/OR Pg ON THE REPLICATION OF  
HSV-2 IN PRIMARY CERVICAL CELLS

A. Effect of a range of concentration of E<sub>2</sub>

| Treatment                                 | Total pfu/cell<br>$\bar{x} \pm \text{S.D.}$ | No. of plates<br>per condition | $\frac{\text{pfu/cell (treated)}}{\text{pfu/cell (control)}}$ |
|---|---|--------------------------------|---|
| HSV-2 alone                               | 10.0 $\pm$ 0.5                              | 3                              | 1.0   |
| HSV-2 + 10 <sup>-9</sup> M E <sub>2</sub> | 10.5 $\pm$ 0.4                              | 3                              | 1.0   |
| HSV-2 + 10 <sup>-8</sup> M E <sub>2</sub> | 9.3 $\pm$ 1.2                               | 3                              | 0.9   |
| HSV-2 + 10 <sup>-7</sup> M E <sub>2</sub> | 10.0 $\pm$ 0                                | 3                              | 1.0   |
| HSV-2 + 10 <sup>-6</sup> M E <sub>2</sub> | 10.0 $\pm$ 0                                | 3                              | 1.0   |

B. Effect of a range of concentrations of Pg

| Treatment                     | Total pfu/cell<br>$\bar{x} \pm \text{S.D.}$ | No. of plates<br>per condition | $\frac{\text{pfu/cell (treated)}}{\text{pfu/cell (control)}}$ |
|-------------------------------|---|--------------------------------|---|
| HSV-2 alone                   | 9.5 $\pm$ 1.0                               | 3                              | 1.0   |
| HSV-2 + 10 <sup>-9</sup> M Pg | 12.0 $\pm$ 1.0                              | 3                              | 1.3   |
| HSV-2 + 10 <sup>-7</sup> M Pg | 11.0 $\pm$ 0.7                              | 3                              | 1.2   |

C. Effect of the combination of E<sub>2</sub> and Pg

| Treatment   | Total pfu/cell<br>$\bar{x} \pm \text{S.D.}$ | No. of plates<br>per condition | $\frac{\text{pfu/cell (treated)}}{\text{pfu/fell (control)}}$ |
|---|---|--------------------------------|---|
| HSV-2 alone   | 10.5 $\pm$ 1.9                              | 3                              | 1.0   |
| HSV-2 + 10 <sup>-9</sup> M E <sub>2</sub>           | 14.0 $\pm$ 0.5                              | 3                              | 1.3   |
| HSV-2 + 10 <sup>-9</sup> M Pg                       | 9.0 $\pm$ 0.9                               | 3                              | 0.9   |
| HSV-2 + 10 <sup>-9</sup> M<br>(E <sub>2</sub> + Pg) | 9.5 $\pm$ 1.4                               | 3                              | 0.9   |

TABLE 3.2

Replication of HSV-2 in ZR-75-1 cells

Effect of E<sub>2</sub> and Pg

|   | Total pfu/cell<br>x ± S.D. | No. plates<br>per condition | pfu/cell (treated)<br>pfu/cell (control) |
|---|----------------------------|-----------------------------|--|
| HSV-2 alone                               | 23 ± 4.0                   | 3                           | 1.0                                      |
| HSV-2 + 10 <sup>-9</sup> M E <sub>2</sub> | 30 ± 6.0                   | 3                           | 1.3                                      |
| HSV-2 + 10 <sup>-8</sup> M E <sub>2</sub> | 28 ± 2.0                   | 3                           | 1.2                                      |
| HSV-2 + 10 <sup>-7</sup> M E <sub>2</sub> | 25 ± 4.0                   | 3                           | 1.1                                      |
| HSV-2 + 10 <sup>-9</sup> M Pg             | 17 ± 0.5                   | 3                           | 0.7                                      |
| HSV-2 + 10 <sup>-8</sup> M Pg             | 23 ± 2.0                   | 3                           | 1.0                                      |
| HSV-2 + 10 <sup>-7</sup> M Pg             | 18 ± 0.5                   | 3                           | 0.8                                      |

ZR-75-1 cells were grown and maintained in 50 mm petri dishes as described in section 3.4.2 prior to the start of the experiment. Cells were treated ± a range of concentrations (see table) of E<sub>2</sub> or Pg for 48h prior to infection with HSV-2. Triplicate dishes of cells were infected with HSV-2 at low multiplicity (1 pfu/cell) for 18h in the continued presence of the same hormones. Cells were harvested, virus extracted and titrated as described for infected primary cervical cells in section 3.3.1. The results are expressed as total pfu/cell ± standard deviation (S.D.) and divided by the control value (i.e. HSV-2 alone) to give relative titre values.

Often the cells were contaminated with fungus, probably resulting from initial exposure to non-sterile conditions at the Pathology Department. This problem was largely overcome with the help of Dr. H. Kitchener who agreed to cut off a piece of cervix from the hysterectomy sample before it was sent to the Pathology Department.

### 3.4            STUDIES IN ZR-75-1 CELLS

#### 3.4.1        Introduction

In light of the difficulties involved in culturing primary cervical cells, it was decided to use an established cell line in which the presence of steroid receptors was well characterized. Since no such cervical cell line was available, the breast cancer cell line, ZR-75-1 (Engel et al., 1978) was chosen to study the interaction of steroid hormones and HSV-2.

#### 3.4.2        Method

ZR-75-1 cells were seeded in 50 mm petri dishes at a density of  $10^6$  cells per dish in DMEM + 10% FCS and allowed to plate down for 18h. The medium was then changed to DMEM + 2% HIDCC-FCS for 2 days prior to hormone treatment. Various concentrations of  $E_2$  or Pg (table 3.2) were given 48h prior to infection and were present throughout the course of infection. The cells were infected at a m.o.i. of 1 pfu/cell for 18h by which time the cells showed characteristic HSV c.p.e. Cells were harvested, virus extracted and titrated as described for primary cervical cells (3.3.1).

#### 3.4.3        Results

ZR-75-1 cells were productively infected (table 3.2) yielding a burst size of 20 pfu/cell. There was no significant difference in virus titre between control and hormone-treated cells.

### 3.5            CONCLUSIONS

Neither physiological ( $10^{-9}\text{M}$ ,  $10^{-8}\text{M}$ ) nor pharmacological ( $10^{-7}\text{M}$ ,  $10^{-6}\text{M}$ ) concentrations of  $\text{E}_2$  or Pg have a significant effect on the growth of HSV-2 in primary cultures of cervical cells or in the breast cancer cell line, ZR-75-1 at low multiplicity of HSV-2 infection.

### 3.6            DISCUSSION

As already described in section C1.5, a limited number of studies have been carried out on the effect of steroid hormones on virus growth both in vivo and in vitro. The in vitro studies often used pharmacological doses of hormones and a wide variety of cell types. For example, Gupta and Rapp (1977) reported an inhibition of HSV-2 induced transformation of  $\text{tk}^-$  mouse cells to the  $\text{tk}^+$  phenotype in the presence of  $10^{-5}\text{M}$   $\text{E}_2$ . Higher concentrations of  $\text{E}_2$  had no effect. Concentrations of  $10^{-5}\text{M}$  are extremely unlikely to occur in vivo. Normal plasma levels of  $\text{E}_2$  are around  $10^{-10}\text{M}$ . Plasma levels of  $\text{E}_2$  can be up to  $5 \times 10^{-7}\text{M}$  if  $\text{E}_2$  is being taken in the form of drugs. Rapp and Turner (1979) reported no significant enhancement of plaque production by HSV-1 or HSV-2 in human embryo fibroblast cells or in primary rabbit kidney cells treated with DES but an increased frequency of tk transformation by HSV-1 and HSV-2 in cells pretreated with DES at concentrations of  $10^{-5}$  to  $10^{-6}\text{M}$ .

No studies on the effect of hormones on the growth of HSV in cervical cells have been reported. In view of the association of HSV-2 with CIN (section C1.3), the influence of hormones in the normal growth and development of cervical tissue (section C1.2) and the increased risk of developing CIN if using the contraceptive pill (a mixture of  $\text{E}_2$  and Pg), (section C1.1.3), it is important to establish whether the growth of HSV in cervical cells is affected by hormones.

The results of the studies presented here show that concentrations of  $\text{E}_2$  or Pg in the range  $10^{-6}$  to  $10^{-9}\text{M}$  do not significantly affect the

growth of HSV-2 either in primary cultures of cervical cells or in the breast cancer cell line, ZR-75-1. Significant differences are generally considered to be of an order of magnitude between "control" and "treated" cells. Such changes were not seen in the studies described here.

Although culturing primary cells is the closest in vitro system available for studying an analogous system in vivo, many other factors may influence the state of the cells in which the virus replicates. For example, it has been shown that stromal cells adjacent to breast epithelial cells secrete growth factors which then act on the neighbouring epithelial cells (Schor et al., 1987). The same may be true of the cervix. Any interaction of stromal and epithelial cells in the cervix is not reproduced in primary culture of cervical explants as described here although it may be possible to develop a feeder-layer culture system to look at this question.





FIG. 4.1

pLW PLASMID CONSTRUCTS

The description of the plasmids is given in the text (section 4.2.1).

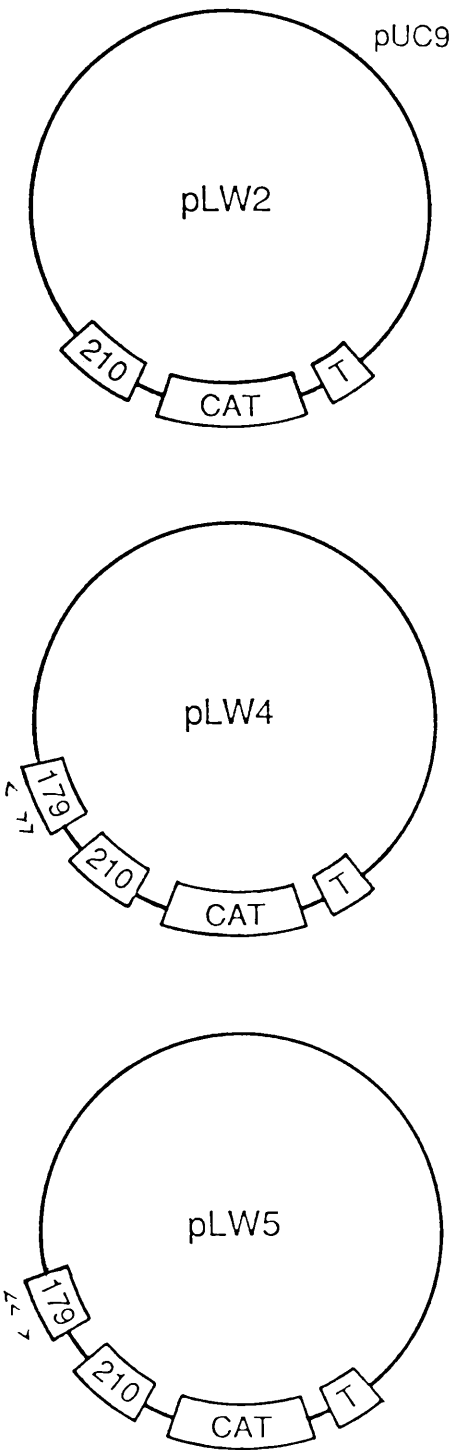


Fig. 4.1 shows the plasmid constructs. The control plasmid is pLW2 consisting of a pUC 9 backbone and the coding sequence of the chloramphenicol acetyl transferase (CAT) gene flanked on the 5'-side by a Sma I/Sau 3A1 fragment of 210 bp from the promoter/leader region of HSV-2 IE mRNA -4/5 and on the 3'-side by a Sma I/Xba I fragment of 100 bp from the terminating sequence of HSV-2 IE mRNA -5. The plasmids pLW4 and pLW5 contain, in addition, a 179 bp Bgl I/Ava II sequence from the intergenic region between IE mRNA's -3 and -4/5 containing three copies of TAATGARAT. The orientation of the central TAATGARAT element in pLW5 is inverted relative to the orientation in pLW4.

#### 4.2.2      Cells

These experiments were done in the ER, PR positive breast cancer cell lines ZR-75-1 and MCF-7. Cells were seeded in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS and allowed to plate down overnight. The medium was changed to DMEM + 2% HIDCC-FCS for 2 days to reduce the level of endogenous steroids to a minimum. Cells were then maintained as controls in DMEM + 2% HIDCC-FCS or as hormone stimulated cells in DMEM + 2% HIDCC-FCS supplemented by the appropriate hormone.

#### 4.2.3      Transfection of Cells and CAT Assay

Modulation of gene expression via the TAATGARAT sequence was assayed by measuring the level of expression of the reporter CAT gene after transfection of pLW2, pLW4 or pLW5 as described in sections 2.2.15 and 2.2.17).

Fig. 4.2 shows the structure of [<sup>14</sup>C]-chloramphenicol. Acetylation can occur at C1 or at C3 or on both C1 and C3. Chloramphenicol is acetylated to different extents depending on the CAT activity of the cell extracts. Results are expressed as n moles acetylated chloramphenicol/μg protein/h and divided by the control value (i.e. CAT expression in the absence of hormones) to allow the relative values to be compared.

### 4.3            ACTIVATION OF TAATGARAT BY SUPERINFECTION WITH UV-tsK

#### 4.3.1        Method

The ability of the HSV virion component, Vmw 65, to modulate gene expression via the TAATGARAT sequence in ZR-75-1 cells was tested. Cells were transfected with 20 µg pLW2 or pLW4 or pLW5. After 36h, the cells were exposed to the trans-inducing virion component by superinfecting the cells with UV-irradiated tsK, which does not transcribe viral genes. (tsK virus was UV-irradiated with an Englehard Hanovia lamp - model 16, operated at 15 watts and 30 mA at a height of 27.5 cm above the sample for 30 min). Cytosol extracts were made and assayed for CAT activity as previously described (sections 2.2.15, 2.2.17).

#### 4.3.2        Results

The results are shown in fig. 4.3 and table 4.1. Superinfection by UV-tsK of cells transfected with pLW4 or pLW5 resulted in approximately 3-fold stimulation of CAT activity compared with non-infected cells. Superinfection of cells transfected with the control plasmid, pLW2, did not stimulate the basal level of CAT activity.

#### 4.3.3        Conclusion

Plasmid directed CAT expression in ZR-75-1 cells is activated 3-fold by the HSV virion when the TAATGARAT sequence is present in either orientation.

#### 4.3.4        Discussion

The levels of CAT activity expressed in pLW4- and pLW5- transformed ZR-75-1 cells were stimulated 3-fold in the presence of the HSV virion supplied by UV-tsK (fig. 4.3 and table 4.1). The virus was UV-irradiated to inactivate transcription. Thus any effect of tsK was due only to the presence of the virion. The plasmids, pLW4 and pLW5 contain TAATGARAT elements in inverse orientation. Thus tsK was able

FIG. 4.3      CAT ASSAY SHOWING ACTIVATION OF TAATGARAT BY UV- $\text{tsK}$   
IN ZR-75-1 CELLS

The experiment was carried out as described in the legend to table 4.1.

The samples were spotted onto TLC paper at the origin (ori). The positions of unconverted chloramphenicol (CAP), chloramphenicol acetylated at C1 (1-ac) and C3 (3-ac) after ascending chromatography and autoradiography are shown.

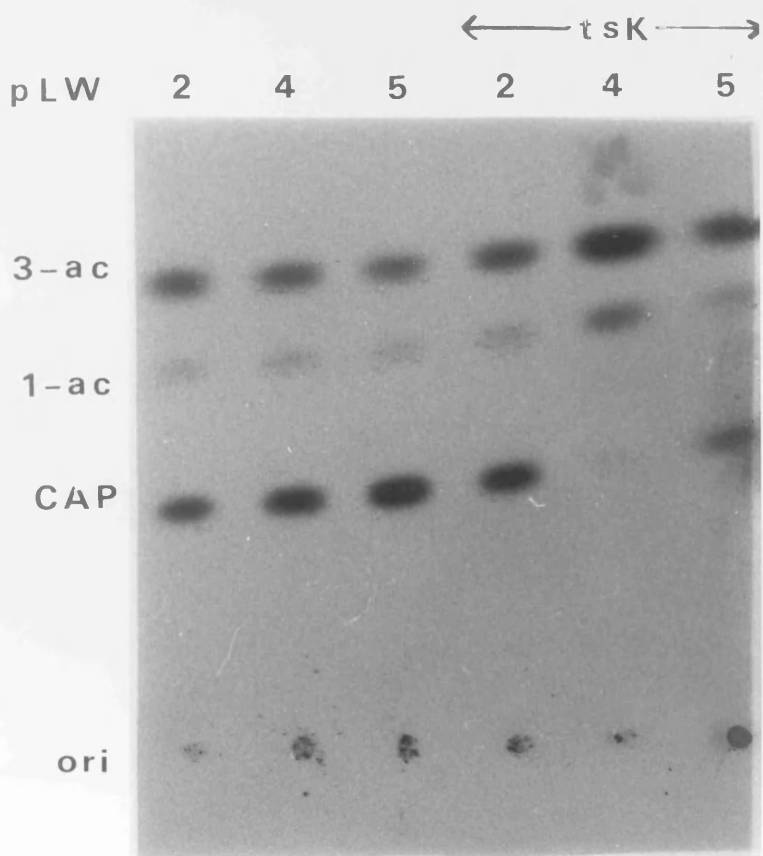


TABLE 4.1

Activation of TAATGARAT by UV-tsK in ZR-75-1 cells

| Treatment             | % [ $^{14}\text{C}$ ]-CM<br>acetylated/<br>assay | [Protein]<br>$\mu\text{g}/\text{assay}$ | Acetylated<br>[ $^{14}\text{C}$ ]-CM<br>$\text{nmoles}/\mu\text{g}/\text{h}$ | Fold<br>Stimulation<br>by UV- <u>tsK</u> |
|-----------------------|--|---|--|--|
| PLW2                  | 48   | 3.90                                    | 1.00   | 1.0                                      |
| PLW4                  | 32   | 3.75                                    | 0.70   | 1.0                                      |
| PLW5                  | 18   | 3.63                                    | 0.41   | 1.0                                      |
| PLW2 + UV- <u>tsK</u> | 40   | 4.55                                    | 0.72   | 0.7                                      |
| PLW4 + UV- <u>tsK</u> | 99   | 4.40                                    | 1.85   | 2.6                                      |
| PLW5 + UV- <u>tsK</u> | 71   | 4.35                                    | 1.34   | 3.3                                      |

ZR-75-1 cells were grown and maintained in 80 cm<sup>2</sup> flasks as described in section 4.2.2. Duplicate flasks of cells were transfected with 20  $\mu\text{g}$  pLW2, pLW4 or pLW5 as described in section 2.2.15. Plasmids are described in section 4.2.1 and illustrated in fig. 4.1. Cells were incubated at 37°C for 36h, then transferred to 38.5°C. One set of flasks was superinfected with UV-tsK (10 pfu/cell) for 12h. The other set of flasks were maintained at 38.5°C. Cells were harvested, cytosol extracts made and assayed for protein concentration and CAT activity as described in sections 2.2.15 - 2.2.17. Results are expressed as n moles acetylated [ $^{14}\text{C}$ ]-chloramphenicol (CM)/ $\mu\text{g}$  protein/h. The "fold" stimulation induced by UV-tsK was calculated relative to the CAT specific activity of the cell extracts not superinfected.

to activate the TAATGARAT element in both orientations. These results agree with those of Gaffney et al. (1985) who found that pLW4- and pLW5-transformed HeLa cells were stimulated 4-fold by superinfection with tsK whereas pLW2- transformed HeLa cells were not stimulated by tsK superinfection.

4.4                    EFFECT OF E<sub>2</sub> AND PROGESTERONE (Pg) ON THE EXPRESSION OF  
pLW2, pLW4 AND pLW5 IN ZR-75-1 AND MCF-7 CELLS

4.4.1                Method

Cells were grown and maintained as described in section 4.2.2 prior to the start of the experiments. Cells were then treated as shown below:

| <u>Treatment</u> | <u>Days 1 and 2</u>               | <u>Days 3 and 4</u>               | <u>Days 5 and 6</u>               |
|------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| Control          | -                                 | -                                 | -                                 |
| E <sub>2</sub>   | -                                 | 10 <sup>-8</sup> M E <sub>2</sub> | 10 <sup>-8</sup> M E <sub>2</sub> |
| Pg               | 10 <sup>-8</sup> M E <sub>2</sub> | 10 <sup>-8</sup> M Pg             | 10 <sup>-8</sup> M Pg             |

Cells were transfected with 20 µg pLW2, pLW4 or pLW5 on day 5 and harvested on day 7.

4.4.2                Results

The results are shown in table 4.2. The majority of experiments were done in ZR-75-1 cells but some experiments were also done in MCF-7 cells. A typical example of a CAT assay result is shown in fig. 4.4.

No consistent modulation of CAT expression by E<sub>2</sub> or Pg was observed. The results varied among experiments as is indicated by some high standard deviations (table 4.2). In some cases, transfection of the pLW plasmids in the presence of hormones led to a small stimulation of CAT expression. However, such increases occurred with pLW2, which does not contain the TAATGARAT element (table 4.2), as well as with



TABLE 4.2

Comparison of the Levels of Expression of pLW2, pLW4 and pLW5  
in the Presence and Absence of Hormones

ZR-75-1 cells (table A) and MCF-7 cells (table B) were grown and maintained in 80 cm<sup>2</sup> flasks as described in section 4.2.2. Cells were treated as described in section 4.4.1. Cytosol extracts were prepared and assayed for protein concentration and CAT activity as described in sections 2.2.15 - 2.2.17. The activity of each cell extract was expressed as n moles acetylated chloramphenicol/ $\mu$ g protein/h. The activities of the hormone treated extracts were expressed relative to the activities of the extracts not treated with hormones (given a value of 1.0). The results in the table show the mean values and standard deviations of several experiments. The number of experiments is shown in brackets.

TABLE 4.2

Comparison of the Levels of Expression of pLW2, pLW4 and pLW5  
in the Presence and Absence of Hormones

A. ZR-75-1 cells

|                           |  |   |
|---------------------------|--|---|
| $\frac{\text{pLW2}}{1.0}$ | $\frac{\text{pLW2} + \text{E}_2}{1.6 \pm 1.3 \text{ (5)}}$ | $\frac{\text{pLW2} + \text{Pg}}{2.2 \pm 2.0 \text{ (5)}}$ |
| $\frac{\text{pLW4}}{1.0}$ | $\frac{\text{pLW4} + \text{E}_2}{1.1 \pm 0.4 \text{ (5)}}$ | $\frac{\text{pLW4} + \text{Pg}}{1.0 \pm 0.6 \text{ (5)}}$ |
| $\frac{\text{pLW5}}{1.0}$ | $\frac{\text{pLW5} + \text{E}_2}{0.8 \pm 0.3 \text{ (5)}}$ | $\frac{\text{pLW5} + \text{Pg}}{0.7 \pm 0.4 \text{ (5)}}$ |

B. MCF-7 cells

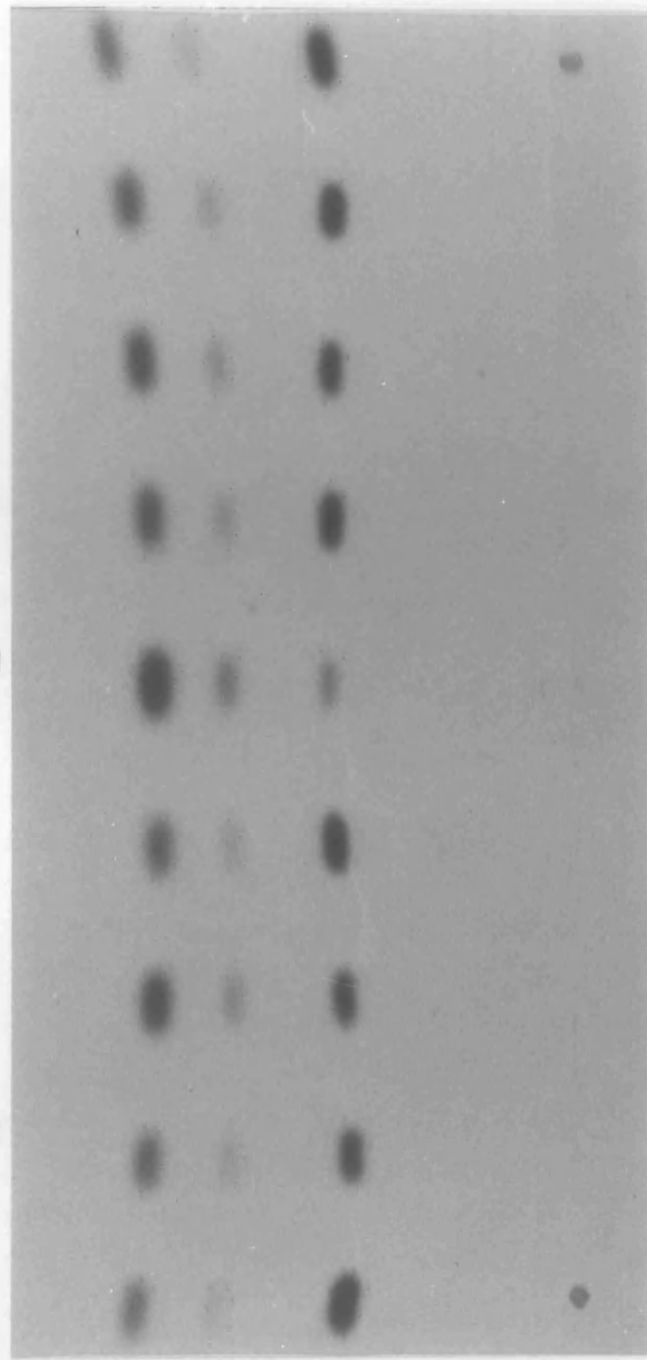
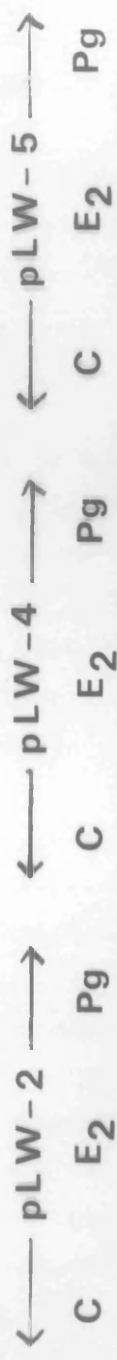
|                           |  |   |
|---------------------------|--|---|
| $\frac{\text{pLW2}}{1.0}$ | $\frac{\text{pLW2} + \text{E}_2}{1.6 \pm 1 \text{ (3)}}$   |   |
| $\frac{\text{pLW4}}{1.0}$ | $\frac{\text{pLW4} + \text{E}_2}{1.4 \pm 0.4 \text{ (3)}}$ | $\frac{\text{pLW4} + \text{Pg}}{2.1 \text{ *}}$ |
| $\frac{\text{pLW5}}{1.0}$ | $\frac{\text{pLW5} + \text{E}_2}{1.0 \pm 0.2 \text{ (3)}}$ | $\frac{\text{pLW5} + \text{Pg}}{1.0 \text{ *}}$ |

\* mean of 2 experiments

FIG. 4.4      CAT ASSAY OF CYTOSOL EXTRACTS FROM ZR-75-1 CELLS  
TRANSFECTED WITH pLW2, pLW4 OR pLW5 IN THE PRESENCE  
AND ABSENCE OF E<sub>2</sub> OR Pg

The experiment was carried out as described in the legend to table 4.2.

The samples were spotted onto TLC paper at the origin (ori). The positions of unconverted chloramphenicol (CAP), chloramphenicol acetylated at C1 (1-ac) and C3 (3-ac) after ascending chromatography and autoradiography are shown.



pLW4, which does contain the TAATGARAT sequence (e.g. fig. 4.4, track 5). Thus any specific effects of the hormones on CAT expression could not be linked to the presence of the HSV activator sequence, TAATGARAT.

#### 4.4.3      Conclusion

In the system described here, there is no evidence for a consistent modulation of gene expression by hormones in the presence of the HSV IE activator sequence, TAATGARAT.

#### 4.4.4      Discussion

The results of section 4.4. show no consistent modulation of CAT activity by E<sub>2</sub> or Pg in the presence of the TAATGARAT element. E<sub>2</sub> induces synthesis of the PR in vivo (Hsueh et al., 1975) and in vitro (Horwitz and McGuire, 1978a). Therefore, cells to be treated with Pg were prestimulated with E<sub>2</sub> to increase the level of PR in the cell. PR, in the presence or absence of its ligand, was not able to activate the TAATGARAT element.

These experiments were dependent on high levels of PR being induced by E<sub>2</sub>, efficient transfection of pLW plasmids and the ability of the PR to recognize the TAATGARAT element in the environment of flanking HSV sequences. In the context of the HSV genome, the activity of the TAATGARAT element is modulated by flanking sequences (Bzik and Preston, 1986). It is perhaps not surprising that the TAATGARAT element, which is only partially homologous to the consensus sequence for the PR binding site in the 5'-flanking region of chicken oviduct genes (section 4.1), is either insufficient by itself or is not flanked by the correct sequences to be recognized by the PR. It may also be that there are different sequence requirements or cellular factors involved in PR recognition of its target sequence in chicken and human cells. It may be that the chromatin configuration of the plasmid sequence is not the same as that required in vivo.

The problem of inducing reproducibly high levels of PR by E<sub>2</sub> could be overcome by cotransfecting an expression vector containing the coding sequence of the PR under the control of a strong promoter along with the pLW plasmids. Such expression vectors have been described for the chicken PR (Conneely et al., 1987; Gronemeyer et al., 1987).

#### 4.5            BINDING OF NUCLEAR PROTEINS IN ZR-75-1 AND MCF-7 CELLS TO TAATGARAT DNA.

##### 4.5.1        Introduction

Vmw 65 interacts indirectly with the TAATGARAT sequence via a cellular protein(s), (Kristie and Roizman, 1987; Preston et al., in press), present in HeLa cells. Since the transfection experiments described in section 4.4 were done in ZR-75-1 and MCF-7 cells, it was decided to investigate if the cellular factor(s) required to form the TAATGARAT/Vmw 65/ cellular protein(s) complex was present in these cells. It was also of interest to know if the presence of E<sub>2</sub> influenced the formation of a TAATGARAT/protein complex. For example, E<sub>2</sub> may increase the level of a cellular factor involved in the interaction with TAATGARAT.

##### 4.5.2        Method

Cells were grown in 850 cm<sup>2</sup> plastic roller bottles in DMEM + 10% FCS. When the cells were approximately 70% confluent, the medium was changed to DMEM + 2% HIDCC-FCS for 2 days. Cells were then maintained as controls, treated with 10<sup>-8</sup>M E<sub>2</sub> for 48h or infected with HSV-2 for 8h at 37°C. Nuclear extracts were prepared as described in section 2.2.32.

Gel retardation assays (section 2.2.33) were used to look at complex formation between nuclear protein from ZR-75-1 and MCF-7 cells and the [ $\gamma$ -<sup>32</sup>P]-labelled oligonucleotide (19 bp, double-stranded) containing one copy of the TAATGARAT element, as shown below:

5'- TTCGTGGTTAATGAGATGCC -3'  
AAGCACCATTACTCTACGG

Binding reactions were done in the presence of 50 mM NaCl and excess non-specific competitor poly dA.dT/poly dG.dC DNA (section 2.2.33.1). DNA/protein complexes were separated from unbound DNA on an 8% polyacrylamide gel (section 2.2.22).

#### 4.5.3 Results

##### 4.5.3.1 Titration of Nuclear Proteins from ZR-75-1 Cells that Complex with TAATGARAT DNA

The results of titrating increasing amounts of nuclear protein from ZR-75-1 control, E<sub>2</sub>-stimulated and HSV-2 infected cells are shown in fig. 4.5. In each case, as more protein is added, more complex is formed as is shown by the increasing intensity of the upper protein/DNA bands on the autoradiograph. Two major bands are seen running close together near the top of the gel and are likely to represent the major cellular protein complex(es) formed with TAATGARAT DNA. Two lower, fainter bands may represent non-specific binding of cellular proteins to TAATGARAT. Alternatively, the presence of minor bands may represent oligomeric complexes or different cellular factors bound to TAATGARAT. No obvious differences in binding pattern were seen in control and E<sub>2</sub>-stimulated cells. An extra band running below the two major bands was seen in the HSV-2 infected track, suggesting the presence of an HSV specific protein in a TAATGARAT/protein complex.

##### 4.5.3.2 Titration of Nuclear Proteins from MCF-7 Cells that Complex with TAATGARAT DNA

The results of titrating increasing amounts of nuclear protein from MCF-7 control and E<sub>2</sub>-stimulated cells are shown in fig. 4.6. In each case, as more protein is added, more complex is formed as is shown by the increasing intensity of the protein/DNA band on the autoradiograph.

FIG. 4.5      TITRATION OF NUCLEAR EXTRACTS FROM ZR-75-1 CELLS  
( $\pm$  E<sub>2</sub> OR HSV-2) WITH TAATGARAT DNA

The experiment was carried out as described in section 4.5.2.

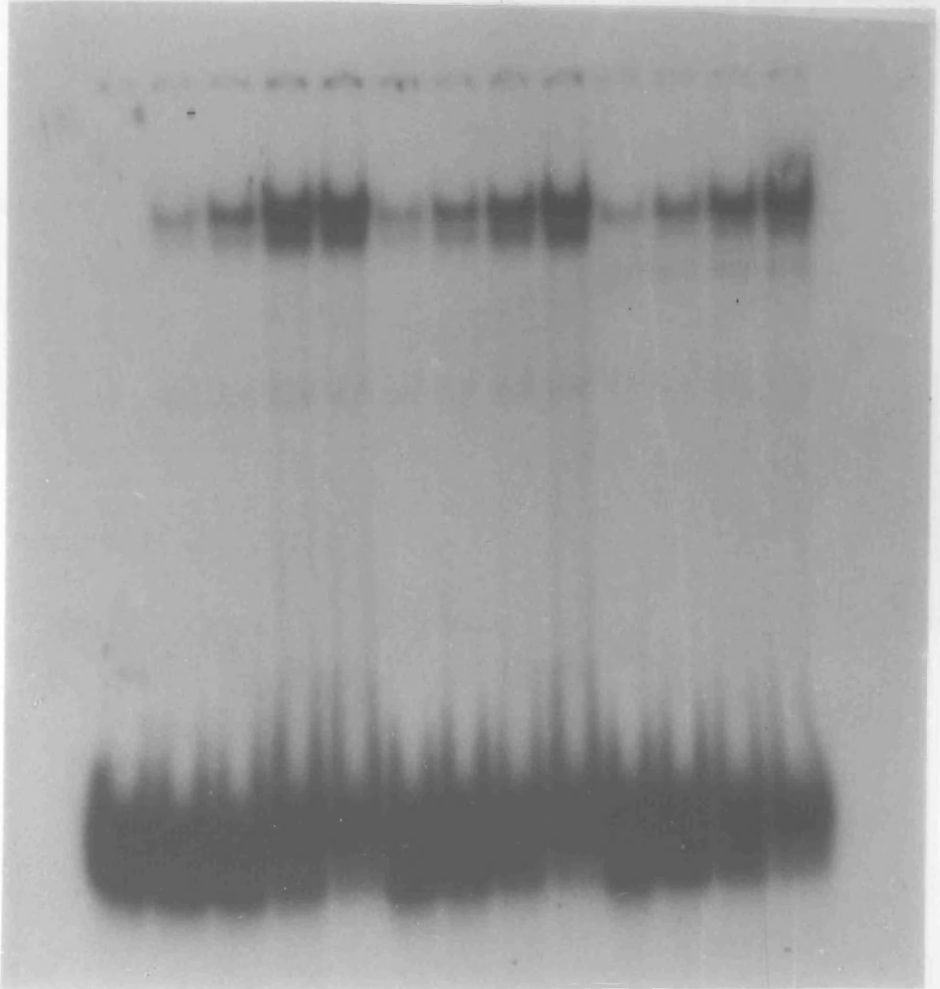


Z R - 7 5 - 1

C

E<sub>2</sub>

HSV-2



0 5 10 20 30 5 10 20 30 5 10 20 30

µg protein

FIG. 4.6      TITRATION OF NUCLEAR EXTRACTS FROM MCF-7 CELLS ( $\pm E_2$ )  
WITH TAATGARAT DNA

The experiment was carried out as described in section 4.5.2.

Only one strong band is seen representing the interaction of TAATGARAT DNA with cellular protein(s). No obvious differences were seen in the binding pattern in control and E<sub>2</sub>-stimulated cell extracts.

#### 4.5.4      Specificity of the TAATGARAT Binding Reaction

##### 4.5.4.1    Method

The ability of excess unlabelled TAATGARAT DNA to compete with radio-labelled TAATGARAT probe for binding to cellular proteins was tested. 20 µg protein extract was incubated with 0.2 ng radiolabelled probe and 0-, 150- or 300- fold excess of unlabelled probe in the DNA binding reaction described in section 2.2.33.1.

##### 4.5.4.2    Results

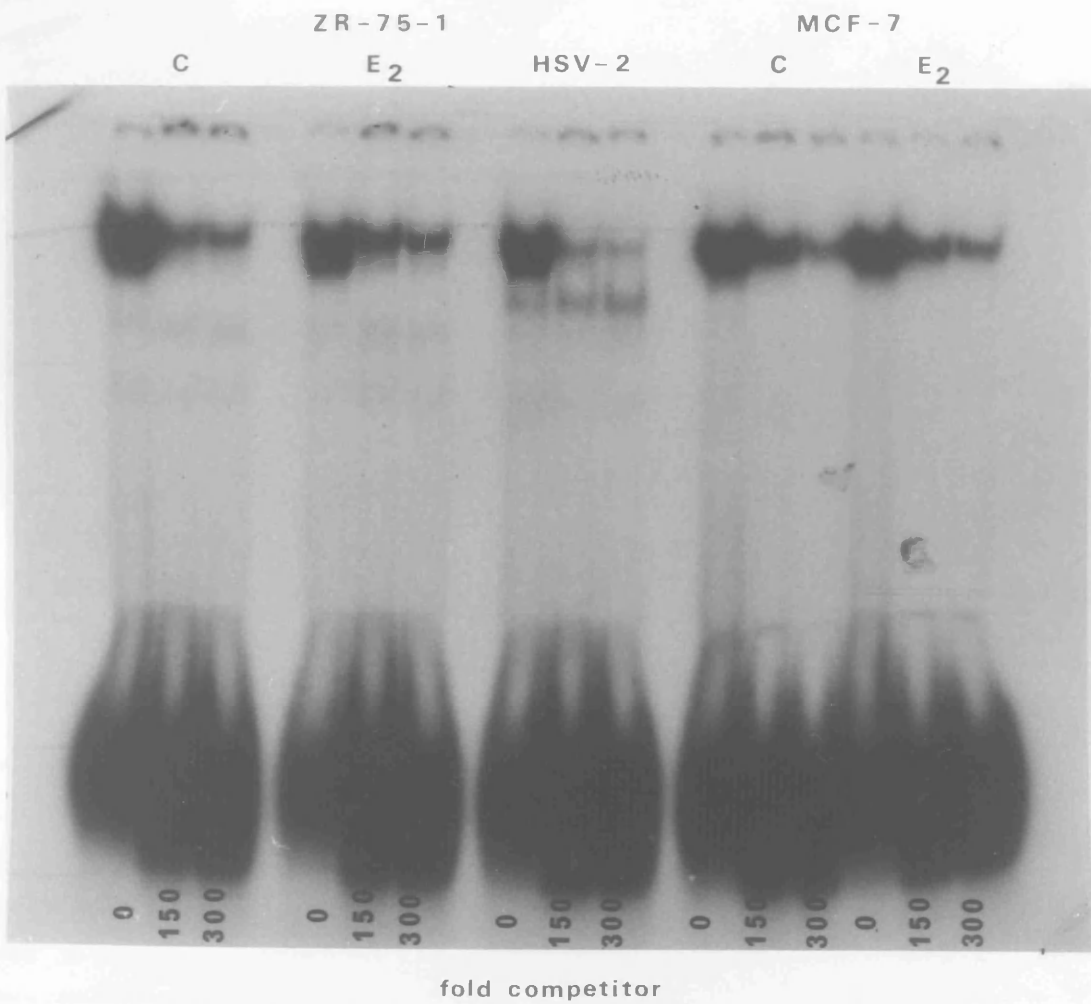
The results are shown in fig. 4.7. Exact resolution of the major band was not accomplished in this gel. However, on the basis of the previous results (figs. 4.5 and 4.6), it is probable that the ZR-75-1 major band is in fact a doublet and the MCF-7 band is a single band. The MCF-7 band appears in the same position on the gel as the top band in the ZR-75-1 extracts (fig. 4.7).

The presence of unlabelled probe greatly reduced the intensity of the top band in MCF-7 and ZR-75-1 extracts (fig. 4.7) indicating that these complexes contain at least some specific protein(s) binding to TAATGARAT. If a second, lower band was present in the ZR-75-1 major complex in the uncompeted tracks, it was completely abolished in the presence of excess unlabelled TAATGARAT DNA, indicating complete specificity for TAATGARAT DNA. The minor faint bands present in each of the ZR-75-1 cell extracts were unaffected by excess unlabelled competing TAATGARAT DNA as was the extra band in HSV-2 infected cell extracts, suggesting that these bands represent non-specific binding.

FIG. 4.7      COMPETITION OF TAATGARAT / PROTEIN BINDING IN ZR-75-1  
AND MCF-7 NUCLEAR EXTRACTS ( $\pm$  E<sub>2</sub> OR HSV-2)

The experiment was carried out as described in section 4.5.4.1

The 'fold' excess competitor unlabelled TAATGARAT DNA is shown at the bottom of the figure.



#### 4.5.5      Conclusions

1. Both ZR-75-1 and MCF-7 cells contain a cellular factor(s) which forms a specific complex with TAATGARAT DNA.
2. The cellular factor(s) involved in binding TAATGARAT DNA is not induced by E<sub>2</sub> or HSV-2.
3. An extra complex forms with HSV infected ZR-75-1 cell extracts compared with control cell extracts but this band is not specific for TAATGARAT DNA.
4. The pattern of protein/DNA complexes formed with ZR-75-1 and MCF-7 extracts are different although both types of cell form a similar major complex with TAATGARAT DNA. More bands are present in ZR-75-1 gels than in MCF-7 gels, indicating the presence of different cellular factors.

#### 4.5.6      Discussion

The results of section 4.5 show competent binding of host cell factors in ZR-75-1 and MCF-7 cells to TAATGARAT DNA. A major, slow-migrating, protein concentration dependent band is seen in gel retardation assays of protein/DNA complexes formed from MCF-7 cell extracts. This band appears as a doublet in ZR-75-1 cell extracts in the gels shown here. A lower percentage polyacrylamide gel would be required to resolve these bands further.

The top band in the ZR-75-1 cell extracts has the same mobility as the major band in the MCF-7 cell extracts. In both cases, this band is competed, although not abolished, by the presence of unlabelled TAATGARAT DNA. The second band in the ZR-75-1 doublet is completely abolished by excess competitor TAATGARAT DNA indicating that this band is totally specific for TAATGARAT DNA. The biological significance of an extra, TAATGARAT specific factor in ZR-75-1 cells compared to MCF-7 cells is unknown. Minor bands present in ZR-75-1 cell extracts are not

competed by excess competitor TAATGARAT DNA and probably represent non-specific binding. MCF-7 cell extracts do not show these minor complexes. This may be due to the presence of different cellular factors in the two cell lines.

The host cell factor(s) which complex with TAATGARAT DNA in ZR-75-1 and MCF-7 cells is not induced by E<sub>2</sub>. HSV-2 infected ZR-75-1 cell extracts form an extra complex not present in control or E<sub>2</sub> treated cell extracts. This complex runs as a band just below the major TAATGARAT bands. However, the band induced in HSV infected extracts is not competed by excess competitor TAATGARAT DNA, indicating that this band does not represent a specific complex with TAATGARAT DNA. Instead, it probably represents binding between some abundant, unidentified viral protein and TAATGARAT DNA.

These results are in agreement with those of Kristie and Roizman (1987) who showed binding of host cell factors to TAATGARAT DNA in uninfected and HSV-1 infected HeLa cell extracts. No specific virus induced complex was observed. In contrast, Preston et al. (in press) have recently identified a novel, virus induced complex consisting of HeLa cell factors, the HSV virion component Vmw 65 and TAATGARAT DNA. This is a large, slow-migrating complex, possibly containing several cellular proteins, which is only seen in low percentage ( $\leq 3.5\%$ ) polyacrylamide gels. This may explain why such a complex was not seen in the system described here using 8% polyacrylamide gels.

The results of TAATGARAT binding studies with E<sub>2</sub> stimulated cell extracts show no effect of E<sub>2</sub> on protein binding to TAATGARAT DNA. It is unlikely therefore that E<sub>2</sub> induces the PR or some other cellular protein to interact with the TAATGARAT sequence. Complex formation in the presence of Pg was not tested. It is possible that although E<sub>2</sub> can induce the PR, the "empty" receptor does not recognize the TAATGARAT sequence but requires the presence of its ligand. However, it seems more likely that the PR simply does not recognize the TAATGARAT sequence since there is only a partial homology between the TAATGARAT sequence and the sequence for binding PR in chicken oviduct responsive genes

(section 4.1) and as both are consensus sequences.

As already discussed (section 4.4.4), the core and/or flanking DNA sequences required for recognition and binding of the human and chicken PR's may be different. Moreover, different cellular factors may be present in the chicken oviduct than in human cells, affecting binding of PR to its target sequence. There may also be a problem of inducing high enough levels of receptor for these binding studies. An expression vector for PR (as previously discussed in section 4.4.4) may help to overcome this problem.



## 5. EFFECT OF E<sub>2</sub> AND HSV ON STEROID RECEPTOR LEVELS

### 5.1 INTRODUCTION

Modulation of cellular ER and PR levels in response to E<sub>2</sub> stimulation and the importance of such changes in the physiological action of oestrogen have been extensively studied in the immature rat uterus and in MCF-7 cells (reviewed by Kassis and Gorski, 1983; see also sections A1.4 and A1.5). Changes in ER and PR levels, induced by E<sub>2</sub>, involve recycling, synthesis and/or processing of the ER. E<sub>2</sub> also induces synthesis of the PR in the rat uterus (Hsueh et al., 1975) and in MCF-7 cells (Horwitz and McGuire, 1978a).

HSV selectively stimulates the expression of certain cellular polypeptides, notably the heat shock proteins (Notarianni and Preston, 1982; LaThangue et al., 1984) as well as other cellular polypeptides whose precise functions have not yet been identified but which may be linked to the process of transformation (Macnab et al., 1985).

Receptors play a central role in steroid hormone action. The ability of HSV to directly modulate the sensitivity of the host cell for E<sub>2</sub> by affecting ER and PR levels was tested in MCF-7 and ZR-75-1 cells.

### 5.2 METHOD

#### 5.2.1 Treatment of Cells

Cells were grown in 175 cm<sup>2</sup> flasks in DMEM + 10% FCS until about 50% confluent. The cells were maintained in serum-free medium for 2 days and thereafter in DMEM + 2% HIDCC-FCS. Cells were then treated as follows:

|                |  |
|----------------|--|
| Control        | Maintained in DMEM + 2% HIDCC-FCS  |
| E <sub>2</sub> | Maintained in DMEM + 2% HIDCC-FCS supplemented by 10 <sup>-8</sup> M E <sub>2</sub> for 48h. |

HSV Maintained in DMEM + 2% HIDCC-FCS for a total of 48h, including the time of virus infection (1 pfu/cell for 16h or 10 pfu/cell for 4 - 6 h).

HSV + E<sub>2</sub> Maintained in DMEM + 2% HIDCC-FCS supplemented by 10<sup>-8</sup>M E<sub>2</sub> for a total of 48h, including the time of virus infection (1 pfu/cell for 16h or 10 pfu/cell for 4 - 6 h).

Cells were harvested and assayed for ER and/or PR as described in sections 2.2.34 - 2.2.37 and outlined briefly below:

#### 5.2.2 "Whole Cell" [<sup>3</sup>H]-Steroid Exchange Assay

Details of this method are given in section 2.2.35.

Whole cell suspensions were prepared in HED buffer containing 1.5 mM MgCl<sub>2</sub> to help maintain the cell integrity. For PR assays, 10% glycerol was added to the buffer to help stabilize the PR. "Two-point" [<sup>3</sup>H]-steroid exchange assays were carried out as full Scatchard analysis required too many cells and was difficult to handle for the number of conditions tested. Steroid bound to receptor was separated from free steroid by Millipore filtration through nitrocellulose filters in the presence of 0.9M NaCl. The specific counts incorporated into [<sup>3</sup>H]-steroid receptor complexes were determined by scintillation counting. The protein content of the cellular suspension was determined by Bio-Rad assay (section 2.2.16). Receptor results were calculated as fmol/mg protein and expressed as a percentage relative to control values.

#### 5.2.3 Cytosol [<sup>3</sup>H]-Steroid Exchange Assay

Cells were harvested and resuspended in HED buffer. Cells were disrupted by sonication and cell debris and nuclei pelleted by centrifugation

(section 2.2.36). A "two-point" [ $^3\text{H}$ ]-steroid exchange assay was set up as described above using cytosol rather than a whole cell suspension. Steroid bound to receptor was separated from unbound steroid by incubation with dextran-coated charcoal which absorbs the free steroid. Results were calculated in a manner similar to that described for the whole cell assay (5.2.2.1).

#### 5.2.4 Abbott ER EIA (Enzyme Immunoassay)

The EIA assays were kindly done by Sheila Cowan or Jackie Courtney in Lab C6, Department of Biochemistry. Details of the procedure are given in the manual accompanying the EIA kit. The anti-ER antibodies are described by Greene et al. (1984). The kit is a "sandwich" assay involving incubation of cell cytosol with beads coated with the ER monoclonal antibody, D547, followed by washing and the addition of a second, different anti-ER antibody (H222 - recognizes an epitope in the steroid - binding domain) to which is attached the peroxidase enzyme for colour development. Samples of cell cytosol were prepared at the same time and in the same way as for the [ $^3\text{H}$ ]-steroid exchange DCC assay. Sonication of cells allowed exposure of the ER antigenic sites for detection by the anti-ER antibody.

### 5.3 RESULTS

#### 5.3.1 Assay of Total ER and PR Levels in ZR-75-1 Cells Treated with $\text{E}_2$ and/or HSV-2

Results from two separate experiments (out of a total of four independent experiments) on ZR-75-1 cells are shown in tables 5.1 and 5.2. Cells were treated with  $10^{-8}\text{M}$   $\text{E}_2$  for 48h and/or infected with HSV-2 for 16h (time used for replication experiments in section 3) at low multiplicity (1 pfu/cell). Whole cell [ $^3\text{H}$ ]-steroid exchange assays were used to measure the relative levels of ER and PR.

The results in table 5.1 show a significant drop in ER level to

TABLE 5.1

Effect of E<sub>2</sub> and/or HSV-2 on Total ER and PR Levels in ZR-75-1  
Cells: Example 1

Growth and pretreatment of cells is described in section 5.2.1.  
Cells were treated as follows (as described in section 5.2.1):

Control      maintained as controls

E<sub>2</sub>            treated with 10<sup>-8</sup>M E<sub>2</sub> alone for 48h

HSV-2        maintained as controls for 32h, then infected with  
HSV-2 (1 pfu/cell) for 16h

HSV-2        treated with 10<sup>-8</sup>M E<sub>2</sub> for 32h, then infected with  
+ E<sub>2</sub>        HSV-2 (1 pfu/cell) for 16h in the continued  
             presence of 10<sup>-8</sup>M E<sub>2</sub>

Cells were harvested and assayed for ER and PR by a "whole cell"  
[<sup>3</sup>H]-steroid exchange assay (section 2.2.35). The protein  
concentrations of the extracts were estimated by Bio-Rad assay  
(section 2.2.16). Receptor concentrations were calculated in  
f mol/mg protein and expressed as percentages of the control  
value (taken as 100%).

The ER results are shown in table A.

The PR results are shown in table B.

TABLE 5.1

Effect of E<sub>2</sub> and/or HSV-2 on total ER and PR levels in ZR-75-1 cells:

Example 1

A. ER Results

| Treatment              | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 195             | 0.50               | 390             | 100                     |
| E <sub>2</sub>         | 18              | 0.69               | 26              | 7                       |
| HSV-2                  | 32              | 0.77               | 42              | 11                      |
| HSV-2 + E <sub>2</sub> | 51              | 1.60               | 32              | 8                       |

B. PR Results

| Treatment              | [PR]<br>fmol/ml | [protein]<br>mg/ml | [PR]<br>fmol/mg | [PR] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 106             | 0.50               | 212             | 100                     |
| E <sub>2</sub>         | 577             | 0.69               | 836             | 394                     |
| HSV-2                  | 39              | 0.77               | 51              | 24                      |
| HSV-2 + E <sub>2</sub> | 358             | 1.60               | 224             | 106                     |

TABLE 5.2

Effect of E<sub>2</sub> and/or HSV-2 on total ER and PR levels in ZR-75-1 cells:

Example 2

A. ER Results

| Treatment              | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 135             | 0.50               | 270             | 100                     |
| E <sub>2</sub>         | 36              | 1.04               | 35              | 13                      |
| HSV-2                  | 22              | 0.52               | 42              | 16                      |
| HSV-2 + E <sub>2</sub> | 23              | 1.43               | 16              | 6                       |

B. PR Results

| Treatment              | [PR]<br>fmol/ml | [protein]<br>mg/ml | [PR]<br>fmol/mg | [PR] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 47              | 0.50               | 94              | 100                     |
| E <sub>2</sub>         | 680             | 1.04               | 654             | 696                     |
| HSV-2                  | 33              | 0.52               | 63              | 67                      |
| HSV-2 + E <sub>2</sub> | 495             | 1.43               | 346             | 368                     |

Cells were treated and results calculated exactly as described in the legend to table 5.1.

approximately 10% of the control value in the presence of E<sub>2</sub> or HSV-2 alone or in combination. The level of PR in the same cells is increased to approximately 400% of the control level by E<sub>2</sub> but is reduced to 20% of the control level by HSV-2 infection. When both E<sub>2</sub> and HSV-2 are present, the level of PR is the same as in the control value. Thus the expected stimulation of PR by E<sub>2</sub> is observed, confirming that the cells are behaving as predicted from previous studies. Yet, PR synthesis is inhibited by HSV-2.

In the second experiment, the same general pattern of response to E<sub>2</sub> and HSV-2 in ZR-75-1 cells is seen (table 5.2). E<sub>2</sub> and HSV-2 alone reduced the level of ER to 13% and 16% respectively. In combination, E<sub>2</sub> and HSV-2 reduced the level of ER further to 6% of the control value. E<sub>2</sub> stimulated the level of PR (700% of control). HSV-2 reduced the level of PR (70% of control) and reduced the increase of PR induced by E<sub>2</sub> (370% of control) when present together with E<sub>2</sub>.

### 5.3.2 Comparison of the Effects of E<sub>2</sub> and HSV-2 on Total ER Levels in ZR-75-1 and MCF-7 Cells

The results of treating ZR-75-1 and MCF-7 cells with 10<sup>-8</sup>M E<sub>2</sub> for 48h and/or infection with HSV-2 at 10 pfu/cell for 4h are shown in table 5.3.

As found previously (tables 5.1 and 5.2), treatment of ZR-75-1 cells with E<sub>2</sub> alone or in combination with HSV-2 caused a drop in ER levels to 10% of the control value (table 5.3A). In this particular experiment, HSV-2 alone only reduced the ER level to 70% of the control value rather than the 10% value found previously (table 5.1).

The results in MCF-7 cells (table 5.3B) show a similar pattern of response to E<sub>2</sub> and HSV-2 as for ZR-75-1 cells (tables 5.1 to 5.3A). E<sub>2</sub> alone caused the level of ER to fall to 10% of the control value. HSV-2 alone resulted in a 50% drop in ER level. The combination of E<sub>2</sub> and HSV-2 reduced the ER level to 20% of the control value.

TABLE 5.3

Comparison of the effects of E<sub>2</sub> and HSV-2 on  
total ER levels in ZR-75-1 and MCF-7 cells

Growth and pretreatment of ZR-75-1 cells (table A) and MCF-7 cells (table B) is described in section 5.2.1. Cells were treated as follows (as described in section 5.2.1):

|                           |  |
|---------------------------|--|
| Control                   | maintained as controls in DMEM + 2% HIDCC-FCS  |
| E <sub>2</sub>            | treated with 10 <sup>-8</sup> M E <sub>2</sub> alone for 48h   |
| HSV-2                     | maintained as controls for 44h, then infected with HSV-2 (10 pfu/cell) for 4h  |
| HSV-2<br>+ E <sub>2</sub> | treated with 10 <sup>-8</sup> M E <sub>2</sub> for 44h, then infected with HSV-2 (10 pfu/cell) for 4h in the continued presence of 10 <sup>-8</sup> M E <sub>2</sub> |

Cells were harvested and assayed for ER by the "whole cell" [<sup>3</sup>H]-steroid exchange assay (section 2.2.35). The protein concentrations of the extracts were estimated by Bio-Rad assay (section 2.2.16). Receptor concentrations were calculated in f mol/mg protein and expressed as percentages of the control value (taken as 100%).

The ER results for ZR-75-1 cells are shown in table A.

The ER results for MCF-7 cells are shown in table B.



TABLE 5.3

Comparison of the effects of E<sub>2</sub> and HSV-2 on  
total ER levels in ZR-75-1 and MCF-7 cells

A. ZR-75-1

| Treatment of<br>ZR-75-1 cells | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|-------------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                       | 284             | 0.88               | 323             | 100                     |
| E <sub>2</sub>                | 31              | 0.90               | 34              | 11                      |
| HSV-2                         | 263             | 1.10               | 239             | 74                      |
| HSV-2 + E <sub>2</sub>        | 35              | 1.40               | 25              | 8                       |

B. MCF-7

| Treatment of<br>MCF-7 cells | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|-----------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                     | 313             | 0.22               | 1423            | 100                     |
| E <sub>2</sub>              | 37              | 0.25               | 148             | 10                      |
| HSV-2                       | 486             | 0.70               | 694             | 50                      |
| HSV-2 + E <sub>2</sub>      | 188             | 0.76               | 247             | 20                      |

### 5.3.3      Conclusions

1. Treatment of cells with  $E_2$  and/or infection with HSV-2 results in a reduction of total ER levels to 10% of the control level.
2. The  $E_2$ -induced stimulation of PR is reduced by HSV-2 infection.
3. HSV-2 infection alone results in a reduction of total PR levels.

### 5.3.4      Discussion

It is possible that the reduction in ER levels seen in response to  $E_2$  and/or HSV infection is due to interference of these agents with the [ $^3H$ ]-steroid exchange assay. For example, the presence of  $E_2$  in the medium of the cells before harvesting may be competing with [ $^3H$ ]- $E_2$  for ER binding. HSV may also be interfering with the exchange of [ $^3H$ ]- $E_2$  and endogenous  $E_2$ . To investigate this further, immunological and biochemical methods of assaying ER were compared in the following experiment (section 5.4).

## 5.4      EFFECT OF $E_2$ AND HSV ON ER LEVELS MEASURED BY IMMUNOLOGICAL AND BIOCHEMICAL METHODS

### 5.4.1      Introduction

To test if the presence of exogenous  $E_2$  in the medium of cells and/or the presence of HSV was interfering with the [ $^3H$ ]-steroid exchange assay, ER levels were measured by the DCC exchange assay and by the Abbott EIA method using a monoclonal antibody to the ER. The antibody should recognize both filled and available receptor.

### 5.4.2      Method

Cells were treated with  $E_2$  and/or HSV as previously described (5.2.1). Cells were disrupted by sonication and the cytosol split into aliquots for the DCC assay, the EIA assay and the Bio-Rad protein assay.

#### 5.4.3      Results

The results of experiments done in ZR-75-1 and MCF-7 cells are shown in tables 5.4 and 5.5 respectively. In all cases,  $E_2$  and HSV-2 caused a reduction in ER levels. However, in the case of  $E_2$  treatment, the loss of ER, measured by the EIA method (approximately 10 - 20 % - see tables 5.4A and 5.5A), was less than when ER levels were measured by the DCC assay (60% in ZR-75-1 cells, table 5.4B; 90% in MCF-7 cells, table 5.5B). In MCF-7 cells (table 5.5), the fall in ER levels induced by HSV-2 was 80% when assayed by either method. However, the results in ZR-75-1 cells (table 5.4) show a 95% fall in ER level measured by the EIA assay and a 70% fall measured by the DCC assay.

The results of a further experiment, including the effect of HSV-1  $\pm E_2$ , HSV-2  $\pm E_2$  or  $E_2$  alone on ER levels are shown in table 5.6. As found in previous experiments, cells treated with  $E_2$ , HSV-2, HSV-2 +  $E_2$  all resulted in a reduction of ER levels when measured by the DCC assay. Table 5.6 extends these findings to include HSV-1 and HSV-1 +  $E_2$  in the ability to reduce ER levels. In this experiment,  $E_2$  was given for 6h, the same length of time as virus infection.

The results presented in table 5.6 confirm the results of table 5.5 and table 5.4 that the ER levels assayed by the EIA method are higher than by the DCC assay. In this particular experiment (table 5.6), no reduction of ER levels after  $E_2$  treatment was seen with the EIA method but a 70% reduction was seen by the DCC method. The marked reduction in ER levels, induced by HSV-1 or HSV-2 was observed using either the DCC assay or the EIA assay. In both assays there was a greater reduction of ER when both HSV (-1 or -2) and  $E_2$  were present together than with either agent alone. In a previous experiment (table 5.1A),  $E_2$ , HSV-2 or  $E_2$  + HSV-2 all gave comparable reductions in ER levels.

#### 5.4.4      Conclusions

1.  $E_2$  and/or HSV (-1 or -2) reduce the level of ER in ZR-75-1 and MCF-7 cells.

TABLE 5.4

Effect of E<sub>2</sub> or HSV-2 on ER levels in ZR-75-1 cells  
assayed by immunological and biochemical methods

Growth and pretreatment of ZR-75-1 cells is described in section 5.2.1. Cells were treated as follows (as described in section 5.2.1):

Control      maintained as controls in DMEM + 2% HIDCC-FCS

E<sub>2</sub>            treated with 10<sup>-8</sup>M E<sub>2</sub> for 48h

HSV-2        maintained as controls for 42h, then infected with  
HSV-2 (10 pfu/cell) for 6h.

Cells were harvested and assayed for ER by the enzyme immunoassay (EIA), (section 2.2.37) and by the dextran-coated charcoal (DCC) [<sup>3</sup>H]-steroid exchange assay (section 2.2.36). The protein concentrations of the extracts were estimated by Bio-Rad assay as described in section 2.2.16. The ER concentrations were calculated in f mol/mg protein and expressed as percentages of the control value (taken as 100%).

The results of the EIA assay are shown in table A.

The results of the DCC assay are shown in table B.

TABLE 5.4

Effect of E<sub>2</sub> or HSV-2 on ER levels in ZR-75-1 cells  
assayed by immunological and biochemical methods

A. EIA Results

| Treatment      | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as %<br>of control |
|----------------|-----------------|--------------------|-----------------|-------------------------|
| Control        | 117             | 0.88               | 133             | 100                     |
| E <sub>2</sub> | 88              | 0.76               | 116             | 87                      |
| HSV-2          | 10.5            | 1.52               | 7               | 5                       |

B. DCC Results

| Treatment      | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as %<br>of control |
|----------------|-----------------|--------------------|-----------------|-------------------------|
| Control        | 58              | 0.88               | 66              | 100                     |
| E <sub>2</sub> | 21              | 0.76               | 28              | 42                      |
| HSV-2          | 30              | 1.52               | 20              | 30                      |

TABLE 5.5

Effect of E<sub>2</sub> or HSV-2 on ER levels in MCF-7 cells  
assayed by immunological and biochemical methods

A. EIA Results

| Treatment      | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|----------------|-----------------|--------------------|-----------------|-------------------------|
| Control        | 495             | 1.47               | 337             | 100                     |
| E <sub>2</sub> | 212             | 0.83               | 255             | 76                      |
| HSV-2          | 61              | 0.91               | 66              | 20                      |

B. DCC Results

| Treatment      | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|----------------|-----------------|--------------------|-----------------|-------------------------|
| Control        | 394             | 1.47               | 268             | 100                     |
| E <sub>2</sub> | 23              | 0.83               | 28              | 10                      |
| HSV-2          | 46              | 0.91               | 51              | 19                      |

Growth and treatment of MCF-7 cells, assay of ER and calculation of results were exactly as described in the legend to table 5.4.

TABLE 5.6

Effect of HSV-1 or HSV-2 infection, alone or in combination with  $E_2$  on ER levels in MCF-7 cells assayed by immunological and biochemical methods

Growth and pretreatment of cells is described in section 5.2.1.  
Cells were treated as follows:

|                  |   |
|------------------|---|
| Control          | maintained as controls in DMEM + 2% HIDCC-FCS                                   |
| $E_2$            | treated with $10^{-8}M$ $E_2$ for 6h  |
| HSV-1            | infected with HSV-1 (10 pfu/cell) for 6h  |
| HSV-2            | infected with HSV-2 (10 pfu/cell) for 6h  |
| HSV-1<br>+ $E_2$ | infected with HSV-1 (10 pfu/cell) for 6h<br>in the presence of $10^{-8}M$ $E_2$ |
| HSV-2<br>+ $E_2$ | infected with HSV-2 (10 pfu/cell) for 6h<br>in the presence of $10^{-8}M$ $E_2$ |

Cells were harvested and assayed for ER by the enzyme immunoassay (EIA) and by the dextran-coated charcoal (DCC) [ $^3H$ ]-steroid exchange assay (sections 2.2.37 and 2.2.36 respectively). The protein concentrations of the extracts were estimated by Bio-Rad assay (section 2.2.16). The ER concentrations are calculated in f mol/mg protein and expressed as percentages of the control value (taken as 100%).

The results of the EIA assay are shown in table A.  
The results of the DCC assay are shown in table B.

TABLE 5.6

Effect of HSV-1 or HSV-2 infection, alone or in combination with E<sub>2</sub> on ER levels in MCF-7 cells assayed by immunological and biochemical methods

A. EIA Results

| Treatment              | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 40              | 0.36               | 111             | 100                     |
| E <sub>2</sub>         | 84              | 0.75               | 112             | 100                     |
| HSV-1                  | 58              | 0.82               | 71              | 64                      |
| HSV-2                  | 52              | 1.03               | 50              | 45                      |
| HSV-1 + E <sub>2</sub> | 43              | 1.13               | 38              | 34                      |
| HSV-2 + E <sub>2</sub> | 47              | 1.22               | 39              | 35                      |

B. DCC Results

| Treatment              | [ER]<br>fmol/ml | [protein]<br>mg/ml | [ER]<br>fmol/mg | [ER] as<br>% of control |
|------------------------|-----------------|--------------------|-----------------|-------------------------|
| Control                | 23              | 0.36               | 64              | 100                     |
| E <sub>2</sub>         | 17              | 0.75               | 23              | 36                      |
| HSV-1                  | 13              | 0.82               | 16              | 25                      |
| HSV-2                  | 2               | 1.03               | 2               | 3                       |
| HSV-1 + E <sub>2</sub> | 7               | 1.13               | 6               | 9                       |
| HSV-2 + E <sub>2</sub> | -               | 1.22               | -               | -                       |



2. ER levels measured by the EIA method are generally higher than ER levels measured by the DCC assay.
3. The reduction in ER levels induced by E<sub>2</sub> is less when measured by the EIA method compared to the DCC method.
4. The reduction in ER levels induced by virus is either the same or less by the EIA method compared to the DCC method.
5. The presence of E<sub>2</sub> together with HSV, in some cases, results in a greater reduction in ER levels than with either E<sub>2</sub> or HSV alone.
6. The ER content of MCF-7 cells is greater than that of ZR-75-1 cells.

#### 5.4.5 Discussion

The results and conclusions from the comparison of immunological and biochemical methods for quantitating the level of ER after E<sub>2</sub> treatment and/or HSV infection suggest that E<sub>2</sub> and possibly also HSV interfere with the [<sup>3</sup>H]-steroid exchange assay used here.

As discussed in section 5.3.4, it is possible that an excess of unlabelled E<sub>2</sub> is present in the E<sub>2</sub>-stimulated cytosol which may compete with exogenous [<sup>3</sup>H]-E<sub>2</sub> in the exchange assay for receptor binding, thus reducing the amount of detectable ER bound to E<sub>2</sub>.

HSV may interfere with the exchange assay by modifying or complexing with the receptor so that the receptor is less able to bind [<sup>3</sup>H]-E<sub>2</sub>. In one case (table 5.4), a higher percentage level of ER after HSV-2 infection was seen with the DCC assay than with the EIA assay. However, the results of other experiments (tables 5.5, 5.6) do not confirm this result.

In each case, the level of ER after E<sub>2</sub> treatment, measured by the EIA assay was greater than by the DCC assay whereas with HSV infection, the levels of ER were the same or different. Therefore HSV and E<sub>2</sub> do not affect the ER in the same way.

The fact that higher levels of ER are detected (even in control cells) by the EIA method compared to the DCC method can be explained by the fact that the EIA method measures both "filled" and "unfilled" receptor and possibly also cross-reacts with antigens other than ER, whereas the DCC assay measures only "unfilled" receptor. MCF-7 cells show a higher endogenous ER level than ZR-75-1 cells by both assays, therefore this result is not due to the fact that the Abbott antibody was raised against the MCF-7 ER.

Interference with the exchange assay by  $E_2$  or HSV does not fully account for the reduction in ER levels. The loss of receptor able to bind steroid after  $E_2$  treatment has been termed "processing" and has been extensively studied in MCF-7 cells (reviewed by Kassiss and Gorski, 1983; see also section A1.4). Horwitz and McGuire (1978c, 1980) described a 70% loss in competent nuclear receptor within 3 - 5 h of  $E_2$  stimulation of MCF-7 cells, not explained by a concomitant rise in cytoplasmic receptor. Induction of PR accompanies loss of ER (Horwitz and McGuire, 1978a) although further studies have shown no direct correlation of PR induction with ER loss (Strobl et al., 1984). Antioestrogens allow accumulation of nuclear receptor complexes but inhibit processing (Horwitz and McGuire, 1978c). It is thought that processing of the ER is intimately associated with the mechanism of action of the ER complex in the nucleus (Kassiss and Gorski, 1983).

The reduction in total ER levels induced by  $E_2$  and/or HSV may be explained by processing of the receptor. Processed ER may still be detected by the EIA monoclonal antibody. Processing in MCF-7 cells involves both physical and kinetic modifications of the receptor, resulting in a less exchangeable form of the receptor (Kassiss et al., 1984). HSV and  $E_2$  may cause similar or different modifications to the receptor. In support of the second possibility, the combination of  $E_2$  and HSV sometimes results in a greater reduction in ER levels than with  $E_2$  or HSV alone (tables 5.2A and 5.6 ). Moreover,  $E_2$  induces the synthesis of PR whereas HSV reduces the level of PR and inhibits the  $E_2$ -induced synthesis of PR (tables 5.1B and 5.2B). Thus  $E_2$  and HSV have different mechanisms of action. HSV may modify the PR in some way to make it less able to bind steroid.

In view of the results of sections 5.3 and 5.4 showing a decrease in the level of the ER protein after  $E_2$  stimulation and HSV-1 or HSV-2 infection, assayed by biochemical and immunological methods, it was decided to test the level of ER by a completely different method. Total cytoplasmic RNA was extracted from  $E_2$  stimulated and virus infected cells, translated in vitro using the rabbit reticulocyte lysate system and the resultant polypeptides analysed by SDS-PAGE.

The method for RNA extraction is described in section 2.2.28.2. The method for in vitro translation is described in section 2.2.29. 1  $\mu$ g total, cytoplasmic RNA was translated in vitro and the amount of [ $^{35}$ S]-methionine incorporated into each sample was taken as a measure of the relative amount of protein synthesized in each case. Equal amounts of total protein (50 000 cpm/sample) were denatured in 1X "boiling mix" (section 2.2.24) in a boiling water bath for 3 min, cooled on ice and loaded onto a 7% single concentration SDS polyacrylamide gel as described in section 2.2.24.

Fig. 5.1 shows the results of SDS-PAGE analysis of in vitro translated RNA from ZR-75-1 cells infected with HSV-2 for 0 - 14 h or stimulated with  $10^{-8}$ M  $E_2$  for 6h and 12h. The accumulation of HSV-2 viral polypeptides [e.g. the major capsid protein (157K), the large subunit of ribonucleotide reductase (136K), the glycoprotein B (118K), the 65K DNA binding protein/virion trans-inducing factor (Vmw 65), the small subunit of ribonucleotide reductase (37.8K)] can be clearly seen. The cellular actin band is also clearly visible (43K). In no case (i.e. control,  $E_2$  stimulated or HSV-2 infected extracts) was any band

FIG. 5.1      IN VITRO TRANSLATION OF mRNA FROM ZR-75-1 CELLS AND  
ANALYSIS OF TRANSLATED PROTEINS BY SDS-PAGE

The experiment was carried out as described in section 5.5.2.

The numbers below the figure refer to the following conditions:

1.            in vitro translation mix with no RNA added
2.            cells maintained as controls in DMEM + 2% HIDCC
3. - 9.       cells infected with HSV-2 for 0, 2, 4, 6, 8, 12 and 14 h
10.           cells treated with  $10^{-8}\text{M}$   $\text{E}_2$  for 6h
11.           cells treated with  $10^{-8}\text{M}$   $\text{E}_2$  for 12h

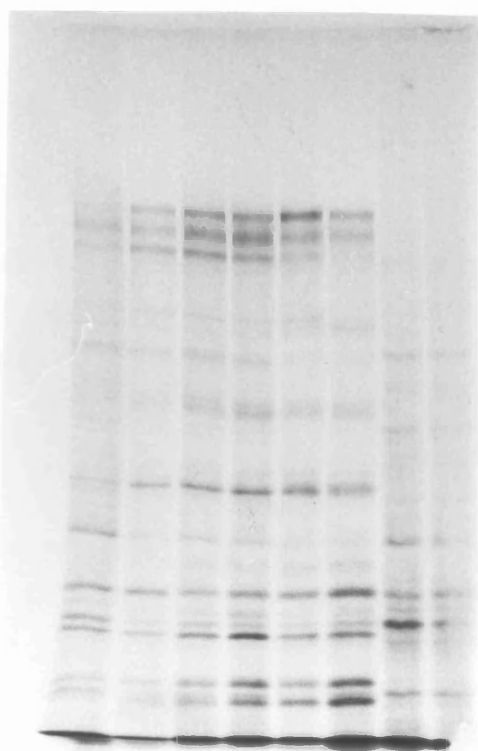
Samples 1. - 3. and samples 4. - 11. were run on separate gels at the same time.

The position of the following proteins on the gel (reading top to bottom) are marked by dashed lines:

major capsid protein (157K)  
large subunit of ribonucleotide reductase (136K)  
glycoprotein B (118K)  
65K<sub>DBP</sub> / Vmw 65  
actin (43K)  
small subunit of ribonucleotide reductase (37.8K)



1 2 3



4 5 6 7 8 9 10 11

corresponding to the ER protein of around 68K mol. wt. seen. Similar experiments were carried out with most of the MCF-7 RNA samples which showed elevated levels of the ER message on Northern blots (section 6). In no case was the ER protein band observed, either when the in vitro translated proteins were subjected to SDS-PAGE or when immunoprecipitated with the same anti-ER monoclonal antibody (H222) as was used in the EIA assays (section 5.4).

(SDS-PAGE and immunoprecipitation experiments were kindly carried out by Dr. J. Macnab).

#### 5.5.4 Conclusion

RNA, extracted from ZR-75-1 or MCF-7 cells and translated in vitro fails to show evidence of the ER protein as judged by SDS-PAGE and immunoprecipitation of the translated proteins.

#### 5.5.5 Discussion

The failure to translate the ER message in vitro by the method described here is not peculiar to the ER. Results of Dr. J. Macnab (personal communication) have shown that in vitro translation of RNA from HSV infected and HSV transformed rat embryo cells does not result in the detection of the cellular 90K and 40K proteins shown, by SDS-PAGE and immunoprecipitation analysis, to be present in protein extracts from the same cells (Macnab et al., 1985). A possible explanation is that the proteins undergo post-translational modification (e.g. glycosylation or phosphorylation) so that the final form of the protein is different from the newly translated protein. Moreover, the antibody used in immunoprecipitation reactions may not recognize both forms of the same protein. This is especially possible in the case of the ER monoclonal antibody, raised against a single epitope. If post-translational modification occurs with the ER then the antibody probably recognizes the modified form, rather than the newly synthesized form of the receptor. Walter et al. (1985) used a combination of four ER

monoclonal antibodies and hybrid-selected ER mRNA for in vitro translation and immunoprecipitation of the ER protein. A protein of approximately 65 000 mol. wt. was observed. The difference between the results of Walter et al. (1985) and the results described above may be due to the low abundance of the ER message in our system and the fact that only one monoclonal antibody was used.

6.1INTRODUCTION

HSV infection increases the expression of a cellular gene at the level of transcription (Kemp et al., 1986) as well as the expression of certain cellular polypeptides (as described in section B1.7.2).

During the course of the present study on the interaction of steroid hormones and HSV, the ER cDNA was cloned and sequenced (Walter et al., 1985; Green et al., 1986). The availability of an ER cDNA clone made it possible to study the effect of HSV infection on the ER at the level of transcription.

6.2PROBES USED TO DETECT THE ER MESSAGE6.2.1Oligonucleotide Probes

Synthetic oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer by Dr. J. McLauchlan, Institute of Virology, Glasgow. Labelling of oligonucleotide probes is described in section 2.2.27.

Oligonucleotide 1

Prior to the availability of a cDNA clone and the complete ER mRNA sequence, an oligonucleotide representing the cDNA sequence of one of the  $\lambda$ OR8 clones, described by Walter et al. (1985), was synthesized and used as a probe for the ER message. Once the ER mRNA sequence was published (Green et al., 1986), the ER mRNA sequence corresponding to the synthetic oligonucleotide was identified as nucleotides +1810 to +1844 in the putative steroid binding domain of the ER. The sequence of oligonucleotide 1 is shown below:

3'-ATG TCG TAC TTC ACG TTC TTG CAC CAC GCG GAG ATA-5'



## Oligonucleotide 2

Once the ER mRNA sequence was published (Green et al., 1986), a longer oligonucleotide (57 bases) representing the cDNA strand of the ER mRNA sequence between nucleotides +258 and +315 in the N-terminal domain of the ER was synthesized. The N-terminal domain is the least conserved among steroid receptors (Green and Chambon, 1986). The use of a longer oligonucleotide from the region least homologous to other steroid receptors as a probe should minimise the risk of non-specific hybridization. The sequence of oligonucleotide 2 is shown below:

3'-CGT AGA CCC TAC CGG GAT GAC GTA GTA TAG GTT CCC TTG CTC GAC CTC  
GGG GAC TTG-5'

### 6.2.2      cDNA Probe - OR3

The plasmid, pOR3 (fig. 6.1) containing the 1.3 kb OR3 cDNA insert (described by Green et al., 1986) cloned into the EcoR1 site of pBR322 was obtained from Professor Pierre Chambon. Purified, nick-translated OR3 DNA (section 2.2.26) was used to probe total mRNA in slot-blots and size fractionated mRNA in Northern blots.

## 6.3      SLOT-BLOTS WITH CRUDE CELL EXTRACTS

### 6.3.1      Introduction

Attempts were made initially to develop a method for looking at ER levels in small numbers of ZR-75-1 or MCF-7 cells with a view to examining the effect of HSV infection on ER mRNA levels in primary cultures of cervical cells.

### 6.3.2      Method

ZR-75-1 or MCF-7 cells were seeded in 50 mm diameter petri dishes at a density of  $10^6$  cells per dish in DMEM + 10% FCS and allowed to adhere overnight. Cells were maintained in DMEM + 2% HIDCC-FCS for 2 days

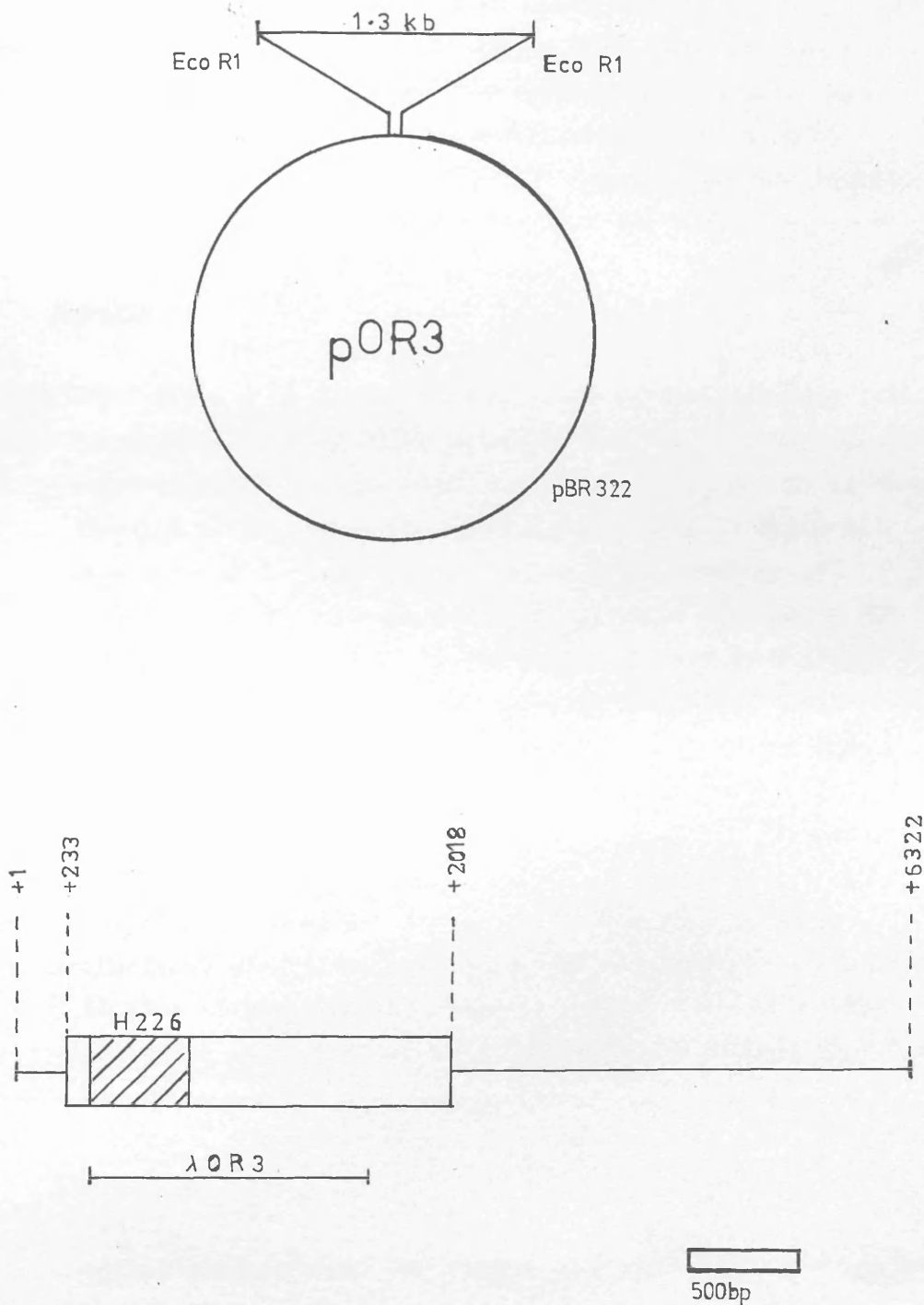
FIG. 6.1      PLASMID pOR3 CONTAINING cDNA INSERT OF ER

The plasmid pOR3 was kindly donated by Professor P. Chambon.  
The 1.3 kb OR3 cDNA fragment is cloned into the Eco RI site of the vector pBR 322.

Below the diagram of the plasmid pOR3, the  $\lambda$ OR3 clone is shown in relation to MCF-7 ER mRNA (reproduced from Green et al., 1986). The MCF-7 ER mRNA is represented by a solid line beginning at nucleotide +1 (the cap site) and ending at nucleotide +6322. The open reading frame is denoted as a box beginning at nucleotide +2018. The region which contains the nucleotide sequence coding for the epitope (in the DNA - binding domain) recognized by the ER monoclonal antibody H226 (Greene et al., 1984) is shown as a hatched box. The  $\lambda$ OR3 clone (which reacts with the H226 antibody) is represented as an arrowed line.

FIG. 6.1

PLASMID pOR3 CONTAINING cDNA INSERT OF ER



to reduce endogenous steroid levels to a minimum. Cells were then maintained as controls, treated with  $E_2$  or infected with HSV-1 or HSV-2.

Crude cellular extracts were prepared by lysing the cells with the detergent NP 40 and spinning down the nuclei and cell debris (2.2.28.1). Equal volumes of cell extract prepared from equal numbers of cells were denatured in formamide/formaldehyde/10X SSC at 65°C for 5 min, then transferred to nitrocellulose membrane via a Schleicher & Schuell slot-blot apparatus (section 2.2.30.1). The membrane was baked at 80°C for 2h prior to hybridization with [ $\gamma$ - $^{32}P$ ]-ATP-labelled oligonucleotide 1 or nick-translated OR3 cDNA (sections 2.2.30.2 and 2.2.30.3).

### 6.3.3 Results

Oligonucleotide 1 probing of a slot-blot of crude cytosol extracts from MCF-7 cells treated with either  $10^{-9}M$   $E_2$  or  $10^{-8}M$   $E_2$  for 24h and 48h and infected with HSV-1 or HSV-2 (5 pfu/cell and 10 pfu/cell) for 8h is shown in fig 6.2. Results of densitometric analysis are shown in table 6.1. The results show a 2- to 3- fold stimulation of the ER message by physiological ( $10^{-9}M$ ,  $10^{-8}M$ ) concentrations of  $E_2$  given for 24h or 48h. Infection of cells with HSV-1 (5 or 10 pfu/cell) resulted in a 2-fold stimulation of the ER message. In cells infected with HSV-2 there was a 2- to 4- fold increase in ER mRNA levels depending on the m.o.i.

### 6.3.4 Conclusions

Preliminary analysis of the effect of HSV on ER mRNA levels, using crude cell extracts on slot-blots indicates that HSV infection stimulates the level of ER mRNA within infected cells to a level similar to that obtained in uninfected cells treated with physiological concentrations of  $E_2$ .

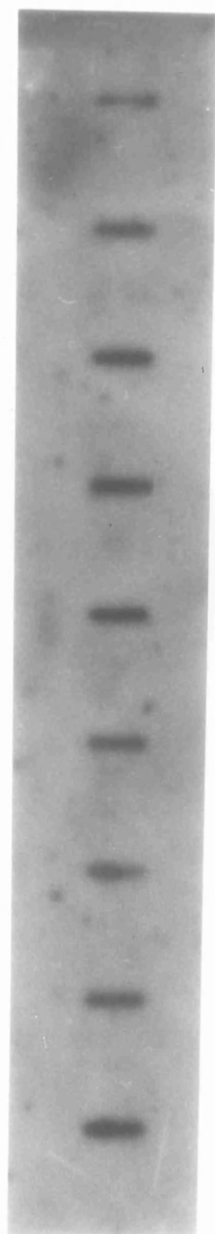
### 6.3.5 Discussion

Although the method used in these experiments was useful for preliminary analysis of ER mRNA levels, it was not considered satisfactory for

FIG. 6.2      SLOT-BLOT OF CRUDE CELL EXTRACTS FROM MCF-7 CELLS TREATED  
 $\pm$  E<sub>2</sub> or HSV-2, PROBED WITH [ $\gamma$ -<sup>32</sup>P]-ATP-LABELLED OLIGO-  
NUCLEOTIDE 1

The experiment was carried out as described in the legend to table 6.1.

MCF-7



control

$10^{-9}$  M  $E_2$  24 h

$10^{-9}$  M  $E_2$  48 h

$10^{-8}$  M  $E_2$  24 h

$10^{-8}$  M  $E_2$  48 h

5 pfu/cell HSV-2

10 pfu/cell HSV-2

5 pfu/cell HSV-2

10 pfu/cell HSV-2

## TABLE 6.1

### Slot-blot analysis of ER mRNA levels in MCF-7 crude cell extracts

Growth and pretreatment of cells is described in section 6.3.2.

Cells were treated as follows:

Control        maintained as controls in DMEM + 2% HIDCC-FCS

E<sub>2</sub>            treated with 10<sup>-8</sup>M or 10<sup>-9</sup>M E<sub>2</sub> for 24h or 48h

HSV            infected with HSV-1 or HSV-2 ( 5 or 10 pfu/cell)  
for 8h

Crude cell extracts were prepared as described in section 2.2.28.1., transferred to nitrocellulose membrane via a slot-blot apparatus and hybridized with [ $\gamma$ -<sup>32</sup>P]-ATP-labelled ER oligonucleotide 1 as described in section 2.2.30.

The autoradiograph was scanned by a Hoefer Scientific GS 300 scanning densitometer and the density of each band was converted to an "area under the curve" using the IBM version of the GS300 data system on an Amstrad PC 1512. The results are recorded in table 6.1 and expressed as percentages of the control (taken as 100%).

TABLE 6.1

Slot-blot analysis of ER mRNA levels in MCF-7 crude cell extracts

| Treatment                      | "Area under<br>the Curve" | <u>Area Sample</u><br><u>Area Control</u> | ER mRNA<br>as % of Control |
|--------------------------------|---------------------------|---|----------------------------|
| Control                        | 28722                     | 1.0                                       | 100                        |
| $10^{-9}$ M E <sub>2</sub> 24h | 55305                     | 1.9                                       | 190                        |
| $10^{-9}$ M E <sub>2</sub> 48h | 81686                     | 2.8                                       | 280                        |
| $10^{-8}$ M E <sub>2</sub> 24h | 69372                     | 2.4                                       | 240                        |
| $10^{-8}$ M E <sub>2</sub> 48h | 72056                     | 2.5                                       | 250                        |
| HSV-1 5pfu/cell                | 48848                     | 1.7                                       | 170                        |
| HSV-1 10pfu/cell               | 56553                     | 2.0                                       | 200                        |
| HSV-2 5pfu/cell                | 58789                     | 2.1                                       | 210                        |
| HSV-2 10pfu/cell               | 115482                    | 4.0                                       | 400                        |



further comparative experiments for the following reasons:

- (a) Although the cell debris and nuclei were spun down after cell lysis, there was the possibility of DNA leaking out of the nuclei into the cytosol thus contaminating the RNA in the cell extract.
- (b) Comparison of mRNA levels was based on the assumption that equal numbers of cells were equally efficiently lysed and yielded comparable amounts of RNA. It was also assumed that each sample was equally efficiently transferred to the nitrocellulose membrane. In fact, in some cases, the presence of protein in the cell extract tended to block the slots on the slot-blot apparatus leading to uneven or incomplete transfer of the sample.

Since the observed differences in ER mRNA levels were relatively small, it was necessary to have a more sensitive and reliable system for analysing these differences and particularly for relating these specifically to the ER message. More refined methods were developed for ZR-75-1 and MCF-7 cells (see next section).

The original reason for using slot-blots to look at RNA levels was that only small numbers of cells were required thus making it a potentially useful system for studying RNA levels in primary cervical cells.

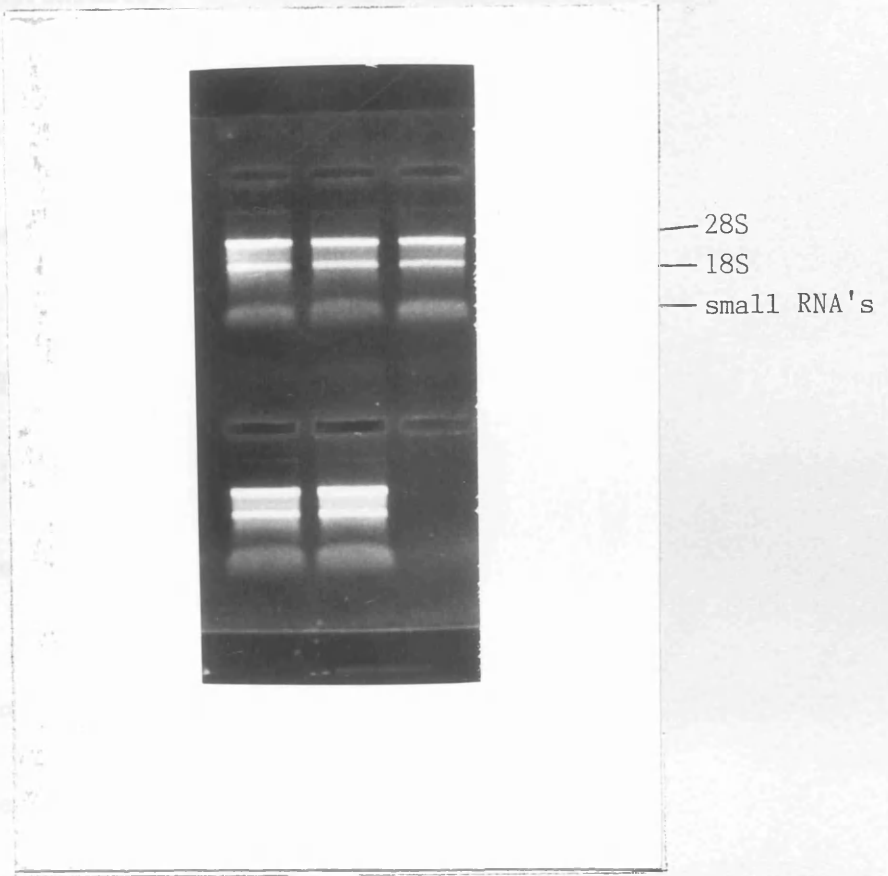
However, difficulties were encountered in culturing cervical cells (described in section 3.3.3). In addition, the method just described for analysing RNA from crude cell extracts was not satisfactory for detailed comparative analysis of RNA levels in cells treated in various ways (for the reasons outlined above). Thus the idea of looking at RNA levels in primary cells was not pursued.

## 6.4            SLOT-BLOTS WITH PURIFIED RNA

### 6.4.1        Method

The above method for analysing RNA levels in crude cell extracts was refined as follows:

- (a) Cells were grown and maintained in 80 cm<sup>2</sup> flasks of cells rather than in 50 mm petri dishes so that RNA could be extracted and purified from the greater number of cells.
- (b) Cells were lysed in an NP 40 containing buffer, cell debris and nuclei were spun down and the cytosol added to a urea/SDS containing buffer to denature the proteins. RNA was purified by three phenol/chloroform extractions followed by ethanol precipitation (section 2.2.28.2).
- (c) RNA was quantitated spectrophotometrically (section 2.2.13).
- (d) To check for DNA contamination and the integrity of the RNA, 1 µg of each sample was electrophoresed on a 1% agarose mini-gel containing ethidium bromide (section 2.2.19). Bands corresponding to 28S, 18S and smaller RNA's were visualized under UV-light and photographed (fig. 6.3). The samples were free of DNA.
- (e) 5 - 10 µg denatured RNA was transferred to nitrocellulose membrane via the slot-blot apparatus.
- (f) The blots were probed either with purified, nick-translated OR3 cDNA or by [ $\gamma$ -<sup>32</sup>P]-labelled oligonucleotide 2 rather than the shorter oligonucleotide 1 to minimize any risk of non-specific hybridization. Probes were labelled to a specific activity of at least  $1 \times 10^8$  cpm/µg.
- (g) Stringent washing conditions for the OR3 cDNA probe were as outlined by Professor Pierre Chambon when he sent us the probe (section 2.2.30.3). Washing conditions for the oligonucleotide probe 2 were as outlined in section 2.2.30.2.



Total cytoplasmic RNA was extracted from MCF-7 cells as described in section 2.2.28.2. The integrity of the RNA was checked by running a small amount (1 - 2  $\mu$ g) of each sample on a 1% agarose mini-gel as described in section 2.2.19. The photograph, shown above, shows distinct bands of 28S and 18S ribosomal RNA and of the smaller RNA species.

## 6.4.2      Results

### 6.4.2.1      Stimulation of ER mRNA by E<sub>2</sub>

Fig. 6.4 and table 6.2 show the results of a time-course of stimulation of ER mRNA by  $10^{-8}\text{M}$  E<sub>2</sub> in ZR-75-1 cells. The level of ER mRNA rose gradually to approximately 3X the control level by 12h, then dropped between 12h and 20h before rising again to 5X the control level by 24h. Fig. 6.5 and table 6.3 show that treatment of MCF-7 cells with  $10^{-8}\text{M}$  E<sub>2</sub> for 24h resulted in a 60% increase in ER mRNA.

In all subsequent experiments involving E<sub>2</sub> stimulation, cells were given  $10^{-8}\text{M}$  E<sub>2</sub> for 24h.

### 6.4.2.2      Stimulation of ER mRNA by HSV-2

In ZR-75-1 cells infected with HSV-2 for 0 - 12 h (fig. 6.6 and table 6.4) there was a gradual increase in ER mRNA levels starting within 2h of infection and rising to 4.5X the control level by 12h post-infection.

In MCF-7 cells infected with HSV-2 for 6 - 8 h there was a 12- to 15-fold stimulation in ER mRNA levels (fig. 6.7 and table 6.5). However, difficulty was experienced in repeating this level of HSV-2 stimulation for unknown reasons.

Cytopathic effect appeared more quickly in MCF-7 cells infected with HSV-1 or HSV-2 than in ZR-75-1 cells infected with HSV-1 or HSV-2. Therefore MCF-7 cells were infected for 6 - 8 h by which time the cells started to detach from their plastic substrate. ZR-75-1 cells could be infected for up to 12h before the cells started to detach from their substrate.

## 6.4.3      Conclusions

1. Purified cytoplasmic RNA was easily quantitated spectrophotometrically and transferred quickly and efficiently to nitrocellulose

FIG. 6.4      SLOT-BLOT OF TOTAL CYTOPLASMIC RNA FROM ZR-75-1 CELLS  
TREATED WITH  $10^{-8}\text{M}$   $\text{E}_2$  FOR 0 - 24 h, PROBED WITH  
 $[\text{}^3\text{}^2\text{P}]$ -LABELLED, NICK-TRANSLATED OR3 cDNA

The experiment was carried out as described in the legend to table 6.2.

ZR-75-1

Stimulation by  $10^{-8}$ M E<sub>2</sub>

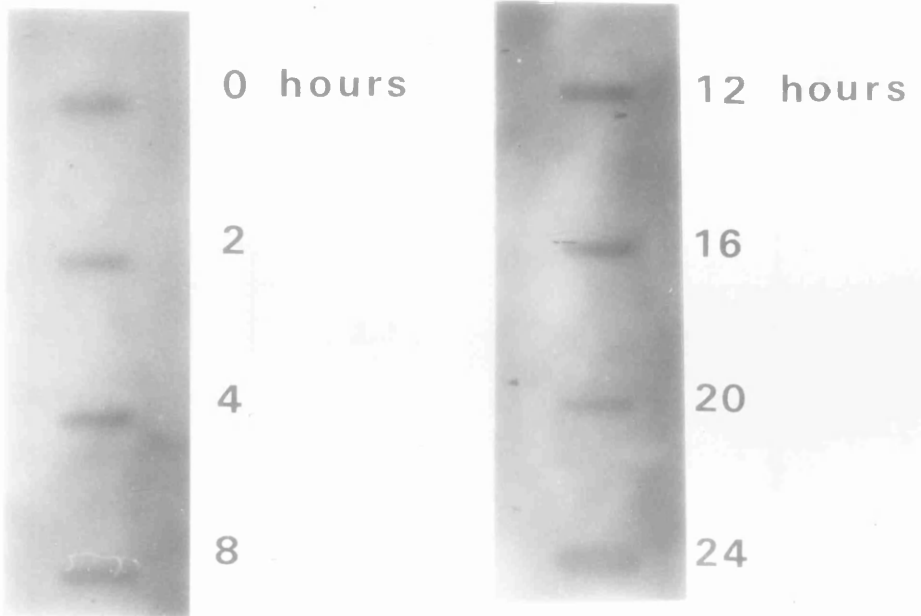


TABLE 6.2

Time-course of stimulation of ER mRNA by  $10^{-8}$  M  $E_2$  IN ZR-75-1 cells

| Time of Stimulation<br>with $10^{-8}$ M $E_2$ (h) | "Area under<br>The Curve" | <u>Area Sample</u><br><u>Area Control</u> | ER mRNA as %<br>of Control |
|---|---------------------------|---|----------------------------|
| 0   | 21521                     | 1.0                                       | 100                        |
| 2   | 20499                     | 1.0                                       | 100                        |
| 4   | 35755                     | 1.7                                       | 170                        |
| 8   | 52562                     | 2.4                                       | 240                        |
| 12  | 58804                     | 2.7                                       | 270                        |
| 16  | 33007                     | 1.5                                       | 150                        |
| 20  | 26597                     | 1.2                                       | 120                        |
| 24  | 104588                    | 4.9                                       | 490                        |

Cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment. The medium was replaced by DMEM + 2% HIDCC-FCS supplemented with  $10^{-8}$  M  $E_2$  for 0 - 24 h. Cytoplasmic RNA was extracted as described in section 2.2.28.2. 10 µg RNA was transferred to nitrocellulose membrane via a slot-blot apparatus and hybridized with [<sup>32</sup>P]-labelled (nick-translated) OR3 cDNA probe as described in section 2.2.30. The autoradiograph was analysed and results were calculated as described in the legend to fig. 6.1.

FIG. 6.5      SLOT-BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
TREATED  $\pm 10^{-8}$ M E<sub>2</sub> FOR 24h, PROBED WITH [<sup>32</sup>P]-LABELLED  
(NICK-TRANSLATED) OR3 cDNA

The experiment was carried out as described in the legend to table 6.3.



TABLE 6.3

Stimulation of ER mRNA levels in MCF-7 cells treated with  $10^{-8}$  M  $E_2$

| Time of Stimulation<br>with $10^{-8}$ M $E_2$ (h) | "Area under<br>the Curve" | <u>Area Sample</u><br>Area Control | ER mRNA as %<br>of Control |
|---|---------------------------|------------------------------------|----------------------------|
| 0   | 8488                      | 1.0                                | 100                        |
| 24  | 13660                     | 1.6                                | 160                        |

Cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment. The medium was replaced by DMEM + 2% HIDCC-FCS supplemented with  $10^{-8}$  M  $E_2$  on one flask of cells for 24h. A second flask of cells was maintained for 24h in DMEM + 2% HIDCC-FCS with no hormone supplement to act as a control. Cytoplasmic RNA was extracted as described in section 2.2.28.2. 10 µg RNA was transferred to nitrocellulose membrane and hybridized with [<sup>32</sup>P]-labelled (nick-translated) OR3 cDNA probe as described in section 2.2.30. The autoradiograph was analysed and results were calculated as described in the legend to table 6.1.

FIG. 6.6      SLOT-BLOT OF TOTAL CYTOPLASMIC RNA FROM ZR-75-1 CELLS  
INFECTED WITH HSV-2 FOR 0 - 12 h, PROBED WITH  
[ $\gamma$ - $^{32}$ P]-ATP-LABELLED ER OLIGONUCLEOTIDE 2

The experiment was carried out as described in the legend to table 6.4.

ZR-75-1



HSV-2

0 hours p.a.

2

4

6

8

12

TABLE 6.4

Time-course of HSV-2 stimulation of ER mRNA in ZR-75-1 cells

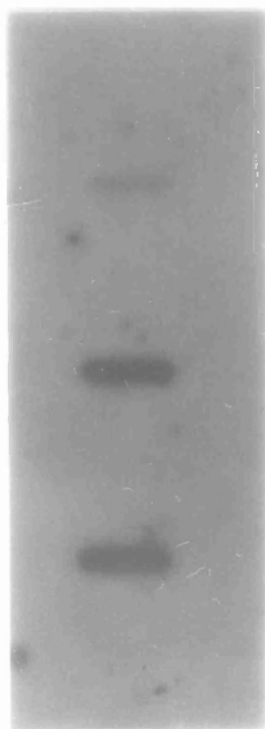
| Time of Infection<br>with HSV-2 (h) | "Area under<br>the Curve" | <u>Area Sample</u><br><u>Area Control</u> | ER mRNA as<br>% of Control |
|-------------------------------------|---------------------------|---|----------------------------|
| 0                                   | 1077                      | 1.0                                       | 100                        |
| 2                                   | 1837                      | 1.7                                       | 170                        |
| 4                                   | 1753                      | 1.6                                       | 160                        |
| 6                                   | 2577                      | 2.4                                       | 240                        |
| 8                                   | 4045                      | 3.8                                       | 380                        |
| 12                                  | 4881                      | 4.5                                       | 450                        |

Cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment. and throughout the course of virus infection. Cells were infected with HSV-2 (10 pfu/cell) for 0 - 12 h. Cytoplasmic RNA was extracted as described in section 2.2.28.2. 10 µg RNA was transferred to nitrocellulose membrane via a slot-blot apparatus and hybridized with [ $\gamma$ -<sup>32</sup>P]-ATP-labelled oligonucleotide 2 as described in section 2.2.30. The autoradiograph was analysed and results were calculated as described in the legend to table 6.1.

FIG. 6.7      SLOT-BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
INFECTED WITH HSV-2 FOR 0, 6 or 8h, PROBED WITH  
[ $\gamma$ - $^{32}$ P]-ATP-LABELLED ER OLIGONUCLEOTIDE 2

The experiment was carried out as described in the legend to table 6.5.

MCF-7



HSV-2

0 hours p.a.

6

8

TABLE 6.5

Stimulation of ER mRNA levels in HSV-2 infected MCF-7 cells

| Treatment | "Area under<br>the curve" | <u>Area Sample</u><br>Area Control |
|-----------|---------------------------|------------------------------------|
| Control   | 412                       | 1                                  |
| HSV-2 6h  | 5125                      | 12                                 |
| HSV-2 8h  | 6314                      | 15                                 |

The experiment was carried out exactly as described in the legend to table 6.4 except that cells were infected with HSV-2 (10 pfu/cell) for 6h or 8h or maintained as a control.

membrane via the slot-blot apparatus.

2. Both E<sub>2</sub> and HSV-2 stimulated the level of ER mRNA to similar extents but over different time intervals, suggesting different mechanisms of action.

#### 6.4.4      Discussion

##### 6.4.4.1    Discussion of Results

The results of slot-blot analysis using purified RNA and either the OR3 cDNA or oligonucleotide 2 as probes confirmed the results obtained with crude cell extracts and the oligonucleotide 1 probe. Both E<sub>2</sub> and HSV-2 were able to stimulate the level of ER mRNA in ZR-75-1 and MCF-7 cells. The high level (12- to 15- fold) of stimulation of ER mRNA induced by HSV-2 infection in MCF-7 cells (fig. 6.7 and table 6.5) was not repeatable in later experiments. However, the fact that experiments with crude cell extracts and purified RNA from MCF-7 cells both showed stimulation of ER mRNA by HSV-2 indicated that these cells were suitable for doing further experiments.

E<sub>2</sub> stimulation of the ER mRNA in MCF-7 cells was greater (2- to 3-fold stimulation) in the slot-blot done with crude cell extracts (fig. 6.2, table 6.1) than with the slot-blot using purified RNA (60% increase), (fig. 6.5, table 6.3). Later experiments in MCF-7 cells using Northern blot analysis (fig. 6.10, table 6.6) showed a 50% increase in ER mRNA in response to E<sub>2</sub> stimulation. Possible reasons for a higher level of stimulation in slot-blots compared to Northern blots are discussed in section 6.6.4.

Slot-blots with purified RNA from ZR-75-1 cells showed a gradual increase in ER mRNA levels in response to HSV-2 infection (fig. 6.6 and table 6.4), reaching a level 4.5X greater than the control value by 12h post-infection. Stimulation of ER mRNA by E<sub>2</sub> was also gradual (fig. 6.4, table 6.2) with maximum levels 5X above the control level after 24h of stimulation.



In the cells used in these experiments, the response to E<sub>2</sub> appeared to be cyclical. The level of ER mRNA increased gradually during the first 12h of E<sub>2</sub> stimulation (2.7-fold) then dropped during the next 8h of E<sub>2</sub> stimulation (down to 1.2-fold) before rising again, reaching 5-fold stimulation by 24h of E<sub>2</sub> treatment. This cyclical effect on the ER mRNA level may be linked to the time of cell division, the time of commitment to DNA synthesis presumably being 12h. Commitment to DNA synthesis in the rat uterus occurs within 12h of E<sub>2</sub> stimulation although maximum levels of DNA synthesis are not reached until 24h (Leake et al., 1975).

Although infection with HSV-2 and stimulation by E<sub>2</sub> gave similar maximum levels of stimulation of ER mRNA, the time-courses were different suggesting that E<sub>2</sub> and HSV act in different ways. The possible mechanisms by which E<sub>2</sub> and HSV affect the level of ER mRNA in the cell are discussed in section 6.6.4.

The reason why cytopathic effect appeared more quickly in MCF-7 cells compared to ZR-75-1 cells infected with HSV-1 or HSV-2 is not clear. However, this observation suggests that there are differences between MCF-7 cells and ZR-75-1 cells even although they were derived from similar sources (Soule et al., 1973, Engel et al., 1978).

#### 6.4.4.2      Discussion of the Advantages and Disadvantages of Slot-Blots

Slot-blots using purified RNA and a specific radiolabelled probe are a quick and easy way of comparing levels of expression of a particular mRNA in a number of different samples at the same time. They are also useful for analysing small quantities (1 - 10 µg) of RNA. The quantity of RNA required depends on the relative abundance of the required message, the specificity of the probe sequence and the specific activity of the probe.

Under the experimental conditions used by Walter et al. (1985), ER mRNA accounted for approximately 0.003% of the total MCF-7 mRNA population. The exact proportion of ER mRNA relative to the total RNA population varies with the stage in the cell cycle. In the system presently being

discussed, 5 - 10 µg total RNA and a probe of specific activity of at least  $1 \times 10^8$  cpm/µg were required to give a strong hybridization signal within 1 - 3 days of exposure to X-ray film. The nick-translated OR3 fragment and the [ $\gamma$ - $^{32}$ P]-labelled oligonucleotide 2 both gave good hybridization signals. However, a shorter exposure time (overnight) to X-ray film was required for blots probed with the oligonucleotide compared to blots probed with the cDNA fragment (2 - 3 days).

The disadvantage of using slot-blots is the inability to check the intact nature of the mRNA and that the probe is hybridizing to a single, specific message of the correct size. Northern blots with molecular weight markers are required to check these factors.

## 6.5 NORTHERN BLOT ANALYSIS OF RNA

### 6.5.1 Standardization of mRNA Levels and Calculation of Results

RNA was extracted, purified and quantitated as described in sections 2.2.28.2 and 2.2.13. 25 µg denatured RNA was electrophoresed on a 1.2% denaturing formaldehyde/MOPS/agarose gel along with RNA molecular weight markers and transferred to Hybond-N membrane as described in sections 2.2.31.1 and 2.2.31.2. The RNA marker track was stained in ethidium bromide and the migration of the bands visualized under UV-light (section 2.2.31.1), (fig. 6.8).

In the slot-blots described in the preceeding section (6.4), RNA was quantitated spectrophotometrically only. It was assumed that the amount of mRNA present in each sample was proportional to the amount of total RNA measured spectrophotometrically. It is advisable, when looking at small differences in mRNA expression, to take as a standard the level of expression of a cellular mRNA which is unlikely to change under the experimental conditions. The actin message is a popular choice for a standard message.

As discussed in section B1.7.1, variable results have been obtained on



— 9.5  
— 7.5  
— 4.4  
— 2.4  
— 1.4  
— 0.3

6  $\mu$ g RNA molecular weight markers (i.e. 1  $\mu$ g of each RNA component) was denatured in formaldehyde/formamide/MOPS at 65°C for 5 min before loading onto a 1.2% denaturing agarose gel (as described in section 2.2.31.1) along with test RNA samples. After electrophoresis, the marker track was cut off and stained in a solution of 0.5  $\mu$ g/ml ethidium bromide overnight. The gel was then destained in distilled water for 1h, visualised under short-wave UV light and photographed.

The size of each RNA component is shown.

the effect of HSV-1 and HSV-2 infection on cellular mRNA levels depending on the cell type and virus strain used. Although certain strains of HSV-2 cause a "shutoff" of cellular mRNA (including actin mRNA) expression (Shek and Bachenheimer, 1985; Bastow et al., 1986), HSV-2 strain HG 52 (the strain used in the present study) has no effect on cellular mRNA expression (Bastow et al., 1986). Although Shek and Bachenheimer (1985) found that HSV-1, strain 17 syn+ shut off actin mRNA expression, there is no evidence in the system described here for this effect in MCF-7 cells with the possible exception of the HSV-1 mutant, tsK (see later results, this section).

There is no evidence for a change in actin mRNA expression in response to E<sub>2</sub>. Thus for the following experiments, actin mRNA was chosen as the standard message against which the level of ER message was compared under various experimental conditions.

To enable multiple probing of a single transfer, the reproducible Hybond-N membrane was used. Blots were first probed for the ER message, then the probe was washed off and the blots reprobed for the actin message (section 2.2.31.6). Each ER mRNA and actin mRNA band was scanned by a Hoefer Scientific GS300 scanning densitometer and converted to an "area under the curve" using the IBM version of the GS350 data system on an Amstrad PC 1512. An example of densitometric traces for ER mRNA and actin mRNA is shown in fig. 6.12(b). The relative amounts of ER mRNA, corrected with respect to the level of expression of actin mRNA, were calculated and compared.

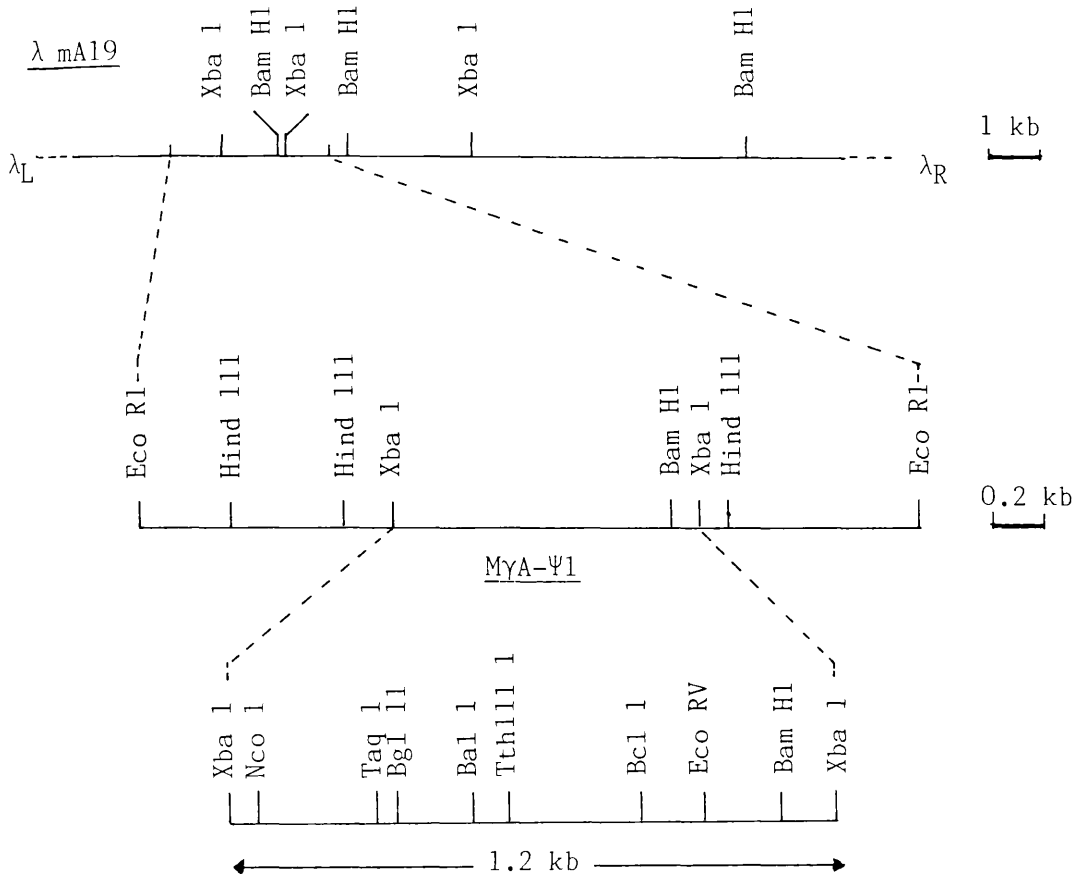
### 6.5.2 Optimization of Hybridization Signals

Hybridization conditions were exactly as described in the Hybond manual and in section 2.2.31.3. Washing conditions were developed to give specific bands corresponding to the actin or ER messages and are described in section 2.2.31.4 and 2.2.31.5.

Optimum signals were obtained with high specific activity probes. The 1.2 kb actin fragment (donated in plasmid form by Dr. D. Leader, Dept. of Biochemistry, Glasgow and shown in fig. 6.9) was purified from its

FIG. 6.9

DESCRIPTION OF ACTIN cDNA PROBE



(Adapted from Leader et al., 1985)

Physical map of recombinant bacteriophage  $\lambda$  mA19 isolated from a mouse genomic library and containing the nucleotide sequence of an actin - like pseudogene (M $\gamma$ A- $\psi$ 1) with an expected 85% identity to the human  $\gamma$  (non-muscle) actin sequence.

The Xba I fragment was cloned into the pUC 13 vector.

vector sequences by restriction enzyme digestion with Xba I and electrophoresis on low melting point agarose (section 2.2.21) and was labelled in the agarose with [<sup>32</sup>P]-dCTP by the oligolabelling method (section 2.2.26.2). Probes of specific activity  $6 \times 10^8$  cpm/ $\mu$ g were routinely obtained, resulting in strong hybridization signals after an overnight exposure to X-ray film. For some unknown reason, OR3 cDNA purified from vector sequences on a low melting point agarose gel and oligolabelled in the same way as actin cDNA, did not hybridize successfully to the ER message. Probably the base composition of the DNA reflects the efficiency of the oligolabelling technique.

OR3 cDNA, purified from its vector on normal agarose (2.2.20) was nick-translated (2.2.26.1) to a specific activity in the range  $5 \times 10^7$  to  $1 \times 10^8$  cpm/ $\mu$ g. Probes of specific activity  $1 \times 10^8$  cpm/ $\mu$ g gave a good hybridization signal after 2 - 3 days exposure to X-ray film. However, probes of lower specific activity resulted in a poorer signal and blots required a longer exposure time. To overcome this problem, the oligonucleotide 2 was used as it was easily labelled and consistently gave strong hybridization signals with the 6.2 kb ER mRNA.

All of the blots shown in the remainder of this section were probed with [ $\gamma$ -<sup>32</sup>P]-labelled ER oligonucleotide 2 first, then with [<sup>32</sup>P]-dCTP oligolabelled actin cDNA.

### 6.5.3      Cells

RNA from MCF-7 cells generally gave stronger hybridization signals than RNA from ZR-75-1 cells, therefore the experiments described in the remainder of this section were all done in MCF-7 cells.

6.6            EFFECT OF HSV INFECTION ALONE OR IN COMBINATION WITH E<sub>2</sub> OR  
CYCLOHEXIMIDE ON ER mRNA LEVELS IN MCF-7 CELLS

6.6.1        Methods

MCF-7 cells were maintained in DMEM + 2% HDCC-FCS for 2 days prior to the start of the experiment. Cells were then treated as follows:

|                        |  |
|------------------------|--|
| Control                | Given fresh medium without any supplements.<br>Harvested with the other cells at the end of the experiment.  |
| E <sub>2</sub> alone   | Given fresh medium containing 10 <sup>-8</sup> M E <sub>2</sub> .<br>Harvested 24h later.  |
| HSV alone              | Given fresh medium without any supplements for 16h.<br>Cells were then infected at a m.o.i. of 10 pfu/cell at 37°C and harvested 8h later.   |
| HSV + E <sub>2</sub>   | Given fresh medium supplemented with 10 <sup>-8</sup> M E <sub>2</sub> for 16h. Cells were then infected at a m.o.i. of 10 pfu/cell at 37°C in the continued presence of 10 <sup>-8</sup> M E <sub>2</sub> and harvested 8h later. |
| HSV +<br>Cycloheximide | Given fresh medium without any supplements for 16h. Cells were then treated with 50 µg/ml cycloheximide for 30 min prior to infecting with virus at a m.o.i. of 10 pfu/cell for 8h in the continued presence of cycloheximide.     |
| <u>tsK</u> alone       | Given fresh medium without any supplements for 16h. Cells were then infected at a m.o.i. of 10 pfu/cell for 8h at 38.5°C.  |

tsK + E<sub>2</sub>                      Same protocol as for HSV + E<sub>2</sub> except that infection was with tsK at 38.5°C.

tsK +                      Same protocol as for HSV + cycloheximide except  
Cycloheximide              that infection with tsK was at 38.5°C.

## 6.6.2              Results

### 6.6.2.1        HSV-1 Infection ± E<sub>2</sub> or ± Cycloheximide

The results of infecting MCF-7 cells with HSV-1 in the presence and absence of E<sub>2</sub> or cycloheximide in two separate experiments are shown in figs. 6.10, 6.11 and tables 6.6, 6.7. The time-course of HSV-1 stimulation of the ER mRNA is shown in fig. 6.12 and table 6.8.

In the first experiment (fig. 6.10 and table 6.6), in cells infected with HSV-1 alone, the level of cytoplasmic ER mRNA rose by approximately 250% compared to the level in control, uninfected cells. In cells treated with E<sub>2</sub> alone, a 50% increase in ER mRNA was observed. Infection with HSV-1 in the presence of E<sub>2</sub> resulted in a 370% increase in ER mRNA which was a greater stimulation than with either HSV-1 or E<sub>2</sub> alone. The presence of cycloheximide during HSV-1 infection inhibited the virus induced increase in ER mRNA levels and caused the level of ER mRNA to fall to 60% of the control level.

In the second experiment (fig. 6.11 and table 6.7), HSV-1 infection resulted in a smaller (100%) increase in ER mRNA than in the first experiment. In this case, infection in the presence of E<sub>2</sub> gave only a slightly greater degree of stimulation (117%) of the ER mRNA than with HSV-1 alone (100% increase). In a similar way to the first experiment, the presence of cycloheximide before and during HSV-1 infection caused the level of ER mRNA to fall to 50% of the control level.

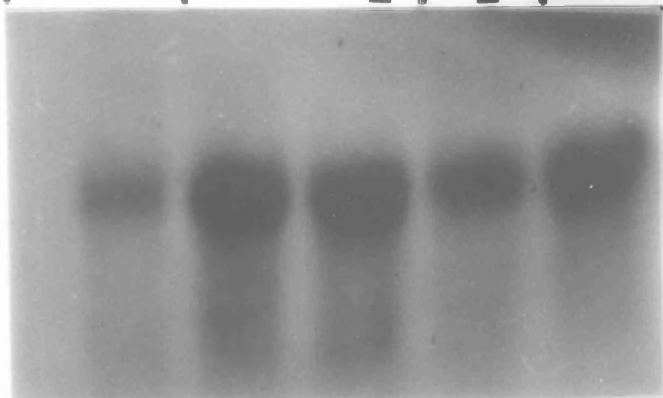
The increase in ER mRNA induced by HSV-1 infection was gradual (fig. 6.12



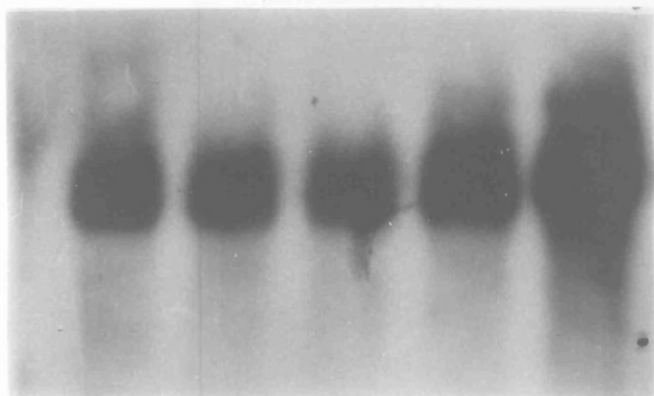
FIG. 6.10    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
INFECTED WITH HSV-1 ALONE, OR IN COMBINATION WITH E<sub>2</sub>  
OR CYCLOHEXIMIDE (CH), PROBED FOR ER mRNA AND ACTIN mRNA

The experiment was carried out as described in the legend to  
table 6.6.

|   | HSV-1 |                |                | HSV-1 |
|---|-------|----------------|----------------|-------|
| C | V     | E <sub>2</sub> | E <sub>2</sub> | CH    |



6 k b



2 k b

TABLE 6.6

Effect of HSV-1 infection alone, or in combination with  
E<sub>2</sub> or cycloheximide, on ER mRNA levels in MCF-7 cells

MCF-7 cells, grown in DMEM + 10% FCS in 80 cm<sup>2</sup> flasks, were maintained in DMEM + 2% HDCC-FCS for 2 days prior to the start of the experiment. Cells were treated with 10<sup>-8</sup>M E<sub>2</sub> alone for 24h or infected with HSV-1 (10 pfu/cell for 8h) alone or in combination with 10<sup>-8</sup>M E<sub>2</sub> or 50 µg/ml cycloheximide (CH) as described in section 6.6.1.

Cytoplasmic RNA was extracted as described in section 2.2.28.2. 25 µg RNA was subjected to Northern blot analysis, hybridized first with [ $\gamma$ -<sup>32</sup>P]-ATP-labelled ER oligonucleotide 2, then with [<sup>32</sup>P]-dCTP-labelled actin cDNA as described in section 2.2.31. The autoradiograph was scanned by a Hoefer Scientific GS 300 scanning densitometer and, using conventional programming, the densities of the bands were converted to "areas under the curve" by an Amstrad PC 1512. The areas were taken as a measure of the relative abundance of the corresponding ER or actin mRNA's. The value for ER mRNA was divided by the value for actin mRNA (standard mRNA), corrected with respect to (wrt) the control value and expressed as a percentage of the control value (taken as 100%).

TABLE 6.6

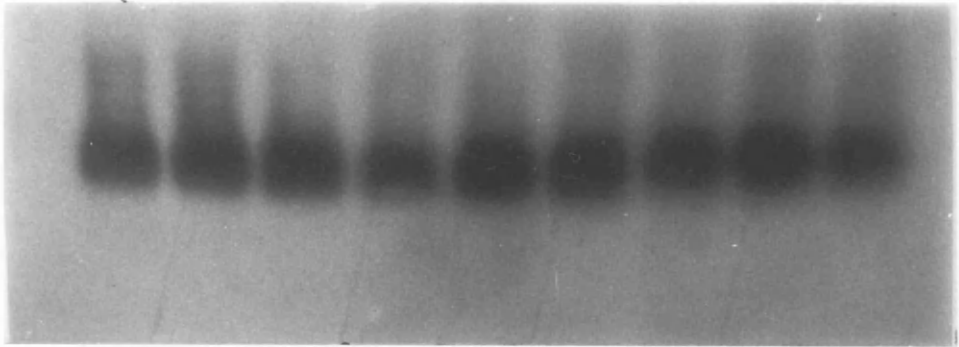
EFFECT OF HSV-1 INFECTION ALONE OR IN COMBINATION WITH  
E<sub>2</sub> OR CYCLOHEXIMIDE ON ERmRNA LEVELS IN MCF-7 CELLS

| Treatment              | ER<br>(Area) | Actin<br>(Area) | ER<br>Actin | Corrected ER<br>(wrt control) | ERmRNA as %<br>of control |
|------------------------|--------------|-----------------|-------------|-------------------------------|---------------------------|
| Control                | 1343         | 1003            | 1.34        | 1.00                          | 100                       |
| HSV-1                  | 4508         | 947             | 4.76        | 3.57                          | 357                       |
| HSV-1 + E <sub>2</sub> | 4022         | 642             | 6.26        | 4.70                          | 470                       |
| E <sub>2</sub>         | 2534         | 1258            | 2.01        | 1.51                          | 151                       |
| HSV-1 + CH             | 4179         | 4893            | 0.85        | 0.64                          | 64                        |

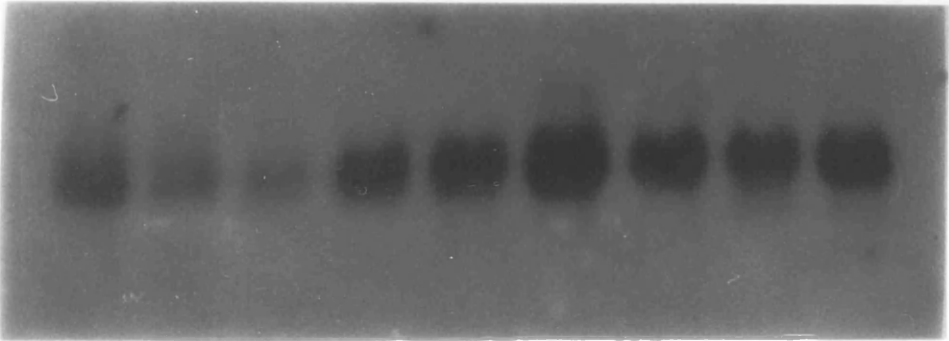
FIG. 6.11    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
INFECTED WITH HSV-1, HSV-2 OR tsK ALONE, OR IN COMBINATION  
WITH E<sub>2</sub> OR CYCLOHEXIMIDE (CH), PROBED FOR ER mRNA AND  
ACTIN mRNA

The experiment was carried out as described in the legend to table 6.7.

|   | tsK |                | HSV-1 |                |    | HSV-2 |                |    |
|---|-----|----------------|-------|----------------|----|-------|----------------|----|
| C | V   | E <sub>2</sub> | V     | E <sub>2</sub> | CH | V     | E <sub>2</sub> | CH |



6 kb



2 kb

TABLE 6.7

Effect of HSV-1, HSV-2 or tsK infection alone, or in combination with E<sub>2</sub> or cycloheximide, in MCF-7 cells

| Treatment                   | ER<br>(Area) | Actin<br>(Area) | <u>ER</u><br>Actin | Corrected ER<br>wrt Control | ER mRNA as<br>% of Control |
|-----------------------------|--------------|-----------------|--------------------|-----------------------------|----------------------------|
| Control                     | 4015         | 2580            | 1.56               | 1.00                        | 100                        |
| <u>tsK</u>                  | 5300         | 944             | 5.61               | 3.61                        | 361                        |
| <u>tsK</u> + E <sub>2</sub> | 4472         | 462             | 9.68               | 6.22                        | 622                        |
| HSV-1                       | 5297         | 1660            | 3.19               | 2.05                        | 205                        |
| HSV-1 + E <sub>2</sub>      | 5767         | 1708            | 3.38               | 2.17                        | 217                        |
| HSV-1 + CH                  | 3107         | 4090            | 0.76               | 0.49                        | 49                         |
| HSV-2                       | 4299         | 2082            | 2.07               | 1.33                        | 133                        |
| HSV-2 + E <sub>2</sub>      | 4920         | 1718            | 2.86               | 1.84                        | 184                        |
| HSV-2 + CH                  | 4304         | 3038            | 1.42               | 0.91                        | 91                         |

MCF-7 cells, grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, were maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment. Cells were infected with tsK at 38.5°C or with HSV-1 or HSV-2 at 37°C (10 pfu/cell for 8h in each case) alone, or in combination with E<sub>2</sub> (10<sup>-8</sup>M for 24h) or cycloheximide (50 µg/ml given for 30 min prior to and throughout the course of virus infection) as described in section 6.6.1. Cytoplasmic RNA was extracted, analysed and the results calculated exactly as described in the legend to table 6.6.

FIG. 6.12 (A)

NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS INFECTED WITH  
HSV-2 (0 - 6 h), HSV-1 (0 - 8 h) OR tsK (8h), PROBED FOR ER mRNA  
AND ACTIN mRNA

The experiment was carried out as described in the legend to table 6.8.

The numbers at the bottom of the figure refer to the time (h) of infection.

The corresponding densitometer traces are shown in fig. 6.12 (B).



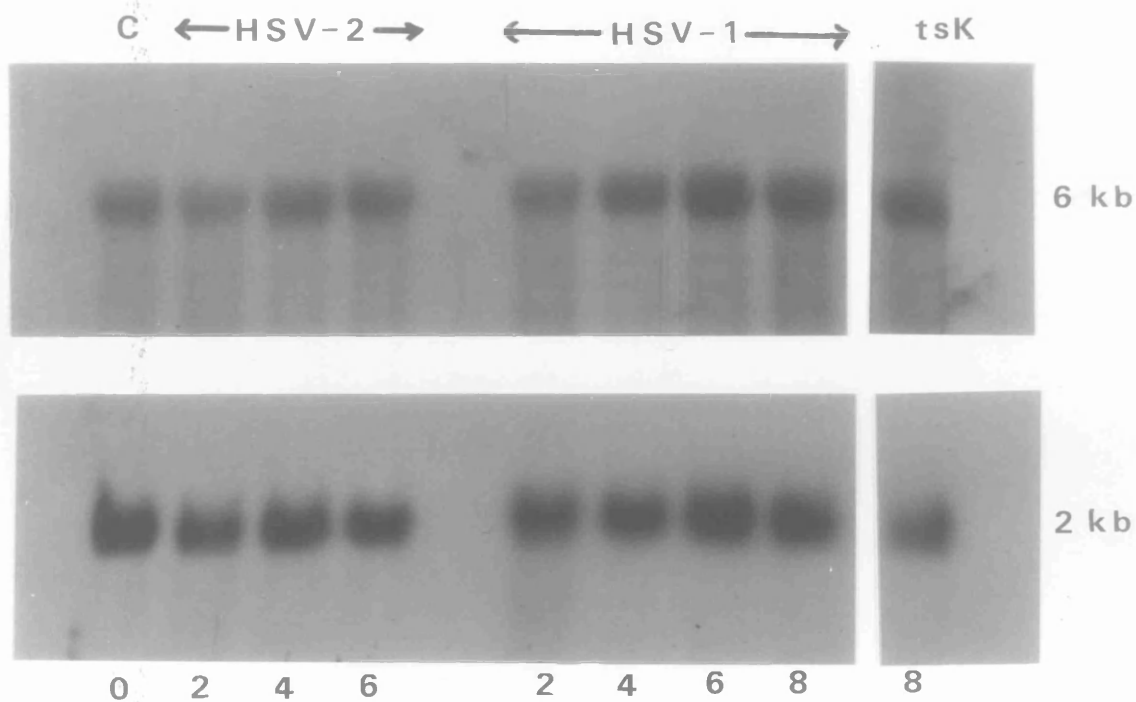


FIG. 6.12 (B)      DENSITOMETRIC TRACES CORRESPONDING TO ER mRNA AND  
ACTIN mRNA BANDS OF NORTHERN BLOT SHOWN IN FIG. 6.12 (A)

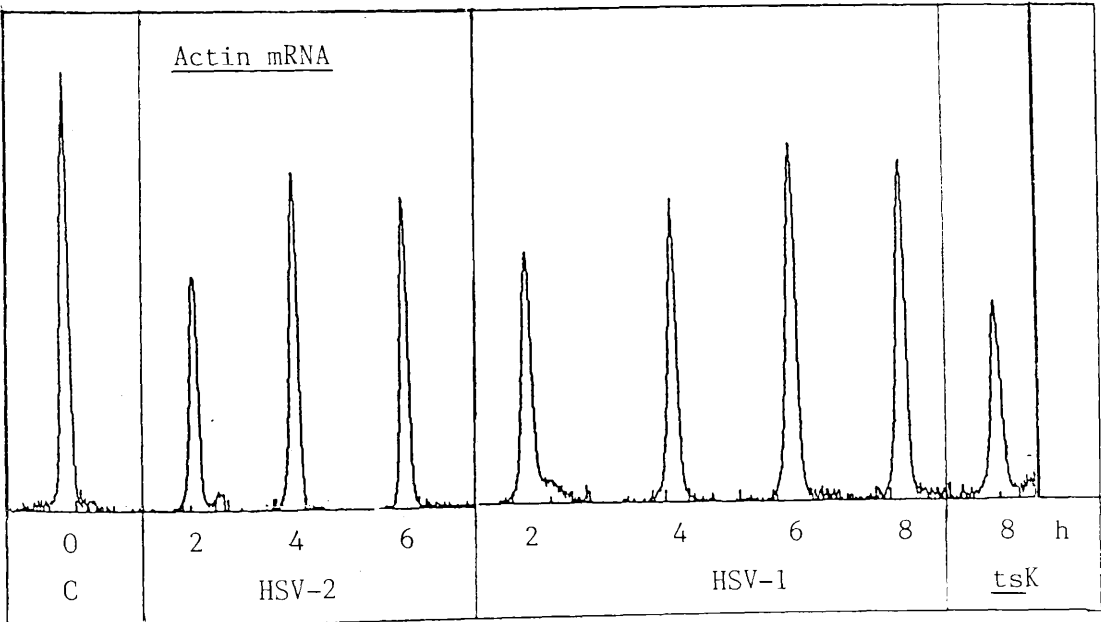
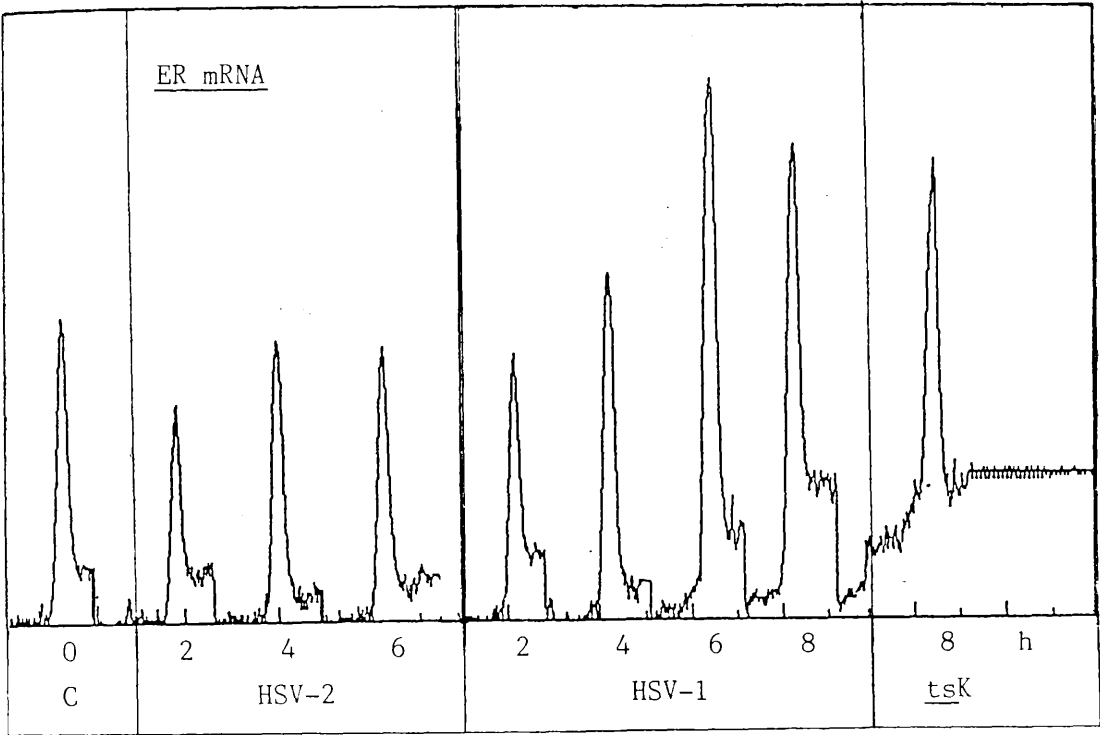


TABLE 6.8

Time-course of ER mRNA stimulation by HSV-1 and HSV-2

| Treatment     | ER<br>(Area) | Actin<br>(Area) | $\frac{\text{ER}}{\text{Actin}}$ | Corrected ER<br>wrt Control | ER mRNA as<br>% of Control |
|---------------|--------------|-----------------|----------------------------------|-----------------------------|----------------------------|
| Control       | 2409         | 3590            | 0.67                             | 1.0                         | 100                        |
| HSV-2 2h      | 1574         | 1982            | 0.79                             | 1.18                        | 118                        |
| HSV-2 4h      | 2128         | 2811            | 0.76                             | 1.13                        | 113                        |
| HSV-2 6h      | 2070         | 2432            | 0.85                             | 1.27                        | 127                        |
| HSV-1 2h      | 2116         | 2650            | 0.80                             | 1.19                        | 119                        |
| HSV-1 4h      | 2742         | 2765            | 0.99                             | 1.48                        | 148                        |
| HSV-1 6h      | 4941         | 3730            | 1.32                             | 1.97                        | 197                        |
| HSV-1 8h      | 4640         | 3404            | 1.36                             | 2.03                        | 203                        |
| <u>tsK</u> 8h | 4719         | 2133            | 2.21                             | 3.29                        | 329                        |

MCF-7 cells, grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS and maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment, were infected with HSV-2 (10 pfu/cell) for 0 - 6 h at 37°C, HSV-1 (10 pfu/cell) for 0 - 8 h at 37°C or tsK (10 pfu/cell) for 8h at 38.5°C or maintained as controls, as described in section 6.6.1.

Cytoplasmic RNA was extracted, analysed and the results calculated as described in the legend to table 6.6.

and table 6.8), starting within 2h of infection (20% increase) and reaching a maximum level by 6 - 8 h post-infection (100% increase, a similar level of stimulation as was found in the second experiment (fig. 6.11; table, 6.7)

#### 6.6.2.2 HSV-2 Infection $\pm$ E<sub>2</sub> or $\pm$ Cycloheximide

The results of infecting MCF-7 cells with HSV-2 in the presence and absence of E<sub>2</sub> or cycloheximide in a single experiment are shown in fig. 6.11 and table 6.7. The time-course of the effect of HSV-2 infection on ER mRNA levels is shown in fig. 6.12 and table 6.8.

HSV-2 infection resulted in a 30% increase in ER mRNA levels by 6 - 8 h post-infection (in the two experiments shown in figs. 6.11, 6.12 and tables 6.7, 6.8). The rise in ER mRNA levels induced by HSV-2 started within 2h of infection (20% increase) and reached 30% stimulation by 6h post-infection (fig. 6.12, table 6.7). At 6h the cells started to detach from their substrate, so that no further time-point was tested. Cells infected with HSV-1 did not detach from their substrate until after 12h of infection. The reason for this difference between HSV-1 and HSV-2 infections is unknown but may reflect the greater instability of the ER mRNA and lesser degree of stimulation seen in HSV-2 infected cells compared to HSV-1 infected cells.

Infection with HSV-2 in the presence of E<sub>2</sub> (fig. 6.11, table 6.7) resulted in a 50% greater increase in ER mRNA levels than with HSV-2 alone. Cycloheximide inhibited the HSV-2 response and the level of ER mRNA fell to 90% of the control value (fig. 6.11, table 6.7).

#### 6.6.2.3 tsK Infection $\pm$ E<sub>2</sub>

The results of two separate experiments on the effect of tsK infection on the level of ER mRNA are shown in figs. 6.11, 6.12 and tables 6.7, 6.8). The effect of the combination of E<sub>2</sub> and tsK in a single experiment is shown in fig. 6.11 and table 6.7.

FIG. 6.13    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
TREATED WITH E<sub>2</sub> OR INFECTED WITH tsK ± E<sub>2</sub>, PROBED FOR  
ER mRNA AND ACTIN mRNA - DEPRESSION OF THE ACTIN MESSAGE  
BY tsK

MCF-7 cells, grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, were maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment. Cells were then treated as follows:

Control        maintained as controls in DMEM + 2% HIDCC-FCS

E<sub>2</sub>            treated with 10<sup>-8</sup>M E<sub>2</sub> for 24h

tsK            infected with tsK at 38.5°C for 8h

tsK + E<sub>2</sub>        treated with 10<sup>-8</sup>M E<sub>2</sub> for 16h, then infected with tsK at 38.5°C for 8h in the continued presence of 10<sup>-8</sup>M E<sub>2</sub>.

Cytoplasmic RNA was extracted and blotted as described in the legend to table 6.6. Densitometric analysis was not carried out due to the weakness of the actin bands.

The results of both experiments on the effect of tsK infection on the level of ER mRNA showed approximately 250% increase in ER mRNA. Infection in the presence of E<sub>2</sub> resulted in a further 260% increase in ER mRNA levels (fig. 6.11, table 6.7).

It is possible that the level of tsK stimulation of ER mRNA calculated in table 6.7 is artifactually high as the actin message is depressed in this experiment. Although such a depression of actin message in response to tsK infection was not always seen (e.g. fig. 6.12, table 6.8), one experiment (fig. 6.13) showed a very significant depression of the actin message in response to tsK infection.

#### 6.6.2.4      Stimulation of ER mRNA by E<sub>2</sub> or tsK in the Absence of Phenol Red

Cells were grown in DMEM + 10% FCS until about 70% confluent and were maintained in Biggar's medium (containing no phenol red) + 2% HIDCC-FCS for 48h prior to the start of the experiment and throughout the course of E<sub>2</sub> stimulation (10<sup>-8</sup>M, 24h) or tsK infection (10 pfu/cell, 8h, 38.5°C).

The results of a single experiment are shown in fig. 6.14 and table 6.9. E<sub>2</sub> stimulation resulted in a 60% increase in ER mRNA levels and infection with tsK resulted in a 150% rise in ER mRNA levels. No effect of tsK on the level of actin mRNA was observed in this case.

#### 6.6.3          Conclusions

1. Infection of MCF-7 cells by HSV-1 results in a gradual rise in ER mRNA levels starting within 2h of infection and, by 8h, reaching maximum levels in the range 100 - 250% (over 3 experiments).
2. Infection of MCF-7 cells by HSV-2 results in a gradual increase in ER mRNA levels starting within 2h of infection and reaching maximum levels of 30% above the control level by 6h post-infection. The significance of a 30% stimulation is unknown but the low value may reflect early detachment of the infected cells from their substrate.

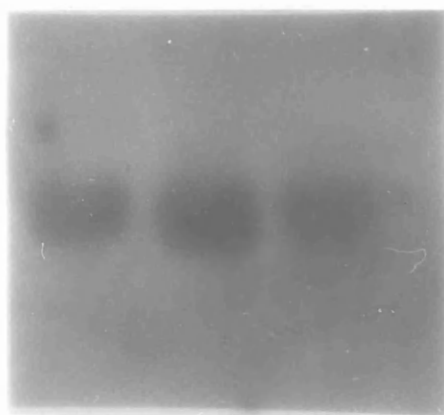
FIG. 6.14    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
TREATED WITH E<sub>2</sub> OR INFECTED WITH t<sub>s</sub>K IN THE ABSENCE OF  
PHENOL RED, PROBED FOR ER mRNA AND ACTIN mRNA

The experiment was carried out as described in the legend to table 6.9.

C E<sub>2</sub> tsK



6 kb



2 kb



TABLE 6.9

Stimulation of ER mRNA by E<sub>2</sub> and tsK in the absence of phenol red

| Treatment                               | ER<br>(Area) | Actin<br>(Area) | $\frac{\text{ER}}{\text{Actin}}$ | ER mRNA as<br>% of Control |
|---|--------------|-----------------|----------------------------------|----------------------------|
| Control                                 | 1655         | 2710            | 0.61                             | 100                        |
| 10 <sup>-8</sup> M E <sub>2</sub> , 24h | 3655         | 3644            | 1.00                             | 164                        |
| tsK, 8h                                 | 3591         | 2335            | 1.54                             | 252                        |

MCF-7 cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in Biggar's medium (containing no phenol red) + 2% HIDCC-FCS for 2 days prior to the start of the experiment and throughout the course of E<sub>2</sub> stimulation (10<sup>-8</sup>M E<sub>2</sub> for 24h) and tsK infection (10 pfu/cell for 8h at 38.5°C). RNA was extracted, analysed and the results calculated as described in the legend to table 6.6.

3. Infection of MCF-7 cells with tsK at 38.5°C for 8h results in approximately 250% stimulation of ER mRNA. In some cases, tsK depresses the actin message.
4. The stimulation of ER mRNA levels by HSV-1, HSV-2 or tsK can be further stimulated in the presence of E<sub>2</sub>.
5. Infection by HSV-1 or HSV-2 in the presence of cycloheximide results in a decrease in the level of ER mRNA to below the control value.
6. Stimulation of ER mRNA by E<sub>2</sub> or virus in the absence of phenol red is of a similar level as is found in the presence of phenol red.

#### 6.6.4      Discussion

##### 6.6.4.1    Discussion of Assay Method

The results obtained with slot-blots and crude cell extracts or purified RNA (section 6.3 and 6.4) on the effect of HSV infection on cellular ER mRNA levels were confirmed by Northern blot analysis. However, the levels of response varied. The increase in ER mRNA induced by E<sub>2</sub> or HSV-2 in the slot-blot experiments were generally higher than those found in Northern blots. In the case of HSV-2 infection, this may be due to the fact that a new stock of virus required to be used for the Northern blot experiments.

It is well known that cultured cells can vary in their response to various inducing agents such as E<sub>2</sub>. It may be that the cells were responding better to E<sub>2</sub> in earlier experiments e.g. in terms of growth rate and macromolecular synthesis. Cells can vary in their behaviour according to the passage number. However, it may be that the differences in response levels were due to the inherent differences in the two systems used to analyse the RNA. Northern blot analysis ensures

that the precise messages are being detected. In the system described here, actin mRNA was taken as a standard message. This was not the case for the slot-blots, therefore it was not possible to correlate increases in ER mRNA with a standard mRNA. This may be one reason for the differing levels of response in Northern and slot-blots. Another possibility is that densitometric scanning of broad bands such as those obtained with Northern blots is not as sensitive as scanning the finer, slot-blot bands. Finally, it may be that some non-specific hybridization was occurring in the slot-blots. With low stringency washing of the ER oligonucleotide or of the OR3 cDNA probe (2X or 1X SSPE at ambient temperature), two bands corresponding to 28S and 18S ribosomal RNA were seen on Northern blots (data not shown).

To test these possibilities, RNA from the same samples used for Northern blots should be used for slot-blots on reproducible Hybond membrane and hybridized with the ER and actin probes under the same hybridization conditions as for the Northern blots.

#### 6.6.4.2 Discussion of Northern Blot Results

The observation that HSV infection in the presence of  $E_2$  results in a greater accumulation of ER mRNA than with either inducing agent alone (figs. 6.10, 6.11 and tables 6.6, 6.7) suggests that the virus and hormone are acting via different pathways. Accumulation of ER mRNA may result from activation of transcription of the ER gene or from stabilization of the message.  $E_2$  induces synthesis of its own receptor (Hsueh et al., 1975, 1976; Kassis and Gorski, 1983) possibly by activation of transcription of the ER gene or by some post-transcriptional control mechanism, perhaps involving stabilization of the ER message or control at the level of translation. By analogy with the control mechanisms worked out for other steroid regulated genes such as the control of MMTV gene expression by glucocorticoid hormones (section A1.7.1.1), it may be that the complex of  $E_2$  with its receptor binds to particular sequences upstream of the ER gene resulting in activation of ER gene transcription. However, currently there is no published information on the 5'-flanking sequence of the human ER gene and it is not known if and how the ER gene is regulated at the transcriptional level.

Occasionally (e.g. fig. 6.11 and table 6.7), E<sub>2</sub> failed to augment the HSV-1 response. This may reflect the state of the cells or it may be that factors other than virus or E<sub>2</sub> are also involved in increasing the level of ER mRNA.

Despite the fact that a component present in commercial preparations of medium containing phenol red has been shown to be a weak oestrogen (Berthois *et al.*, 1986; Rajendran *et al.*, 1987), the presence or absence of phenol red did not affect the level of response to E<sub>2</sub> (fig. 6.14; table 6.9). The level of response to tsK was less (150% increase) in the absence of phenol red than previous experiments done in the presence of phenol red (200 - 300% increase). However, this is unlikely to be due to the absence of phenol red as it would be expected that in the absence of the weak oestrogen, the response would be greater than in the presence of the weak oestrogen. It is more likely that the cells did not respond so well on that occasion to tsK infection.

Induction of ER mRNA levels by virus was inhibited by cycloheximide (figs. 6.10, 6.11 and tables 6.6, 6.7). In fact, the level of ER mRNA fell to between 50% and 60% of the control level in cells infected with HSV-1 in the presence of cycloheximide (figs. 6.10, 6.11 and tables 6.6, 6.7). Cycloheximide was given for 30 min prior to virus infection and was present throughout the course of infection (as already described in section 6.6.1). These results suggest that viral and cellular proteins are important for the induction of ER mRNA levels. The situation may be analogous to the interaction of Vmw 65 with its target TAATGARAT sequence which occurs in association with cellular proteins (sections B1.6.3 and 4.5). A viral protein could trans-activate the ER gene by interacting directly or indirectly (via a cellular factor) with regulatory sequences upstream of the ER mRNA start of transcription. Alternatively, the ER message may be stabilized by a viral and/or cellular protein.

The mutant virus tsK, contains a ts lesion in IE gene 3 such that IE Vmw 175 is inactive and thus IE proteins, negatively regulated by Vmw 175, are overproduced and later classes of proteins are not synthesized at the non-permissive temperature of 38.5°C (Preston, 1979a, Watson and Clements, 1980). Infection with tsK at 38.5°C resulted in an increase of ER mRNA to at least a similar or greater level than with wild-type virus (figs. 6.11, 6.12 and tables 6.7, 6.8), suggesting that IE proteins are sufficient for the effect on ER mRNA levels or that the virion itself plays a role in stimulation of the ER message. However, it should also be noted that in certain experiments (e.g. figs. 6.11, 6.13 and table 6.7) the level of actin mRNA was depressed by tsK infection. This is especially true for the case of tsK + E<sub>2</sub> (fig. 6.11, track 3), which would account for the corresponding high level of stimulation of ER mRNA calculated using actin as the standard message. The actin bands in fig. 6.13 were too faint to trace on the densitometer. Thus although the actin message is a suitable standard for HSV-1, HSV-2 infection and E<sub>2</sub> stimulation, it is not ideal for tsK infection.

The mutant virus, tsK, synthesizes an abnormal form of the Vmw 175 protein (Preston, 1979b) and is known to show some abnormal characteristics compared to wild-type virus. For example, induction of the heat shock response in tsK infected cells (Notarianni and Preston, 1982) results from the production of abnormal forms of the Vmw 175 protein (Russell et al., 1987) rather than the overproduction of IE proteins.

The variation in the level of depression of the actin message may result from possible variation in incubation temperature. The tsK virus is temperature sensitive and the mutant form of Vmw 175 is only synthesized at the non-permissive temperature of 38.5°C. As a communal incubator was used for the tsK experiments, it is possible that an inadvertant drop in temperature may have occurred if the incubator was opened during the course of the experiment.

## 6.7            EFFECT OF HSV IE PROTEINS Vmw 175 AND Vmw 110 ON ER mRNA LEVELS

### 6.7.1        Introduction

As described in section Bl.6.4, the HSV IE proteins, Vmw 110 and Vmw 175 are regulators of gene expression. Since the results of section 6.6 suggest the involvement of a viral protein(s) in stimulation of ER mRNA levels, the ability of Vmw 175 and Vmw 110 to activate the level of expression of the ER message was tested.

### 6.7.2        Plasmids used in Transfection Studies

The following plasmids were kindly supplied by Dr. R. Everett, Institute of Virology:

|         |                 |   |
|---------|-----------------|---|
| pl11    | (Everett, 1987) | encodes IE protein Vmw 110  |
| pl75    | (Everett, 1987) | encodes IE protein Vmw 175  |
| ptk-CAT | (Everett, 1986) | encodes the CAT gene under the control of the HSV early promoter, tk. |

In addition, the plasmid pAT (Twigg and Sheratt, 1980) was used to equalise the total amount of transfected DNA and as a control plasmid.

### 6.7.3        Method

Cells maintained in DMEM + 2% HIDCC-FCS were transfected with pl75 or pl11 or the control plasmid, pAT and harvested 48h later. The relative level of ER mRNA in each case was compared.

As a positive control to show that the transfections were working, the same plasmids were cotransfected with the tk-CAT plasmid and the level of CAT activity was measured 48h later.

FIG. 6.15    CAT ASSAY OF CYTOSOL EXTRACTS FROM MCF-7 CELLS COTRANS-  
FECTED WITH tk-CAT + p175 / p111 / p175 + p111 / pAT

The experiment was carried out as described in the legend to table 6.10.

The samples were spotted onto TLC paper at the origin (ori). The positions of unconverted chloramphenicol (CAP), chloramphenicol acetylated at C1 (1-ac) and C3 (3-ac) after ascending chromatography and autoradiography are shown.

← tk-CAT →

pAT      p175      p111      p175  
   p111

3-ac  
1-ac  
CAP  
ori

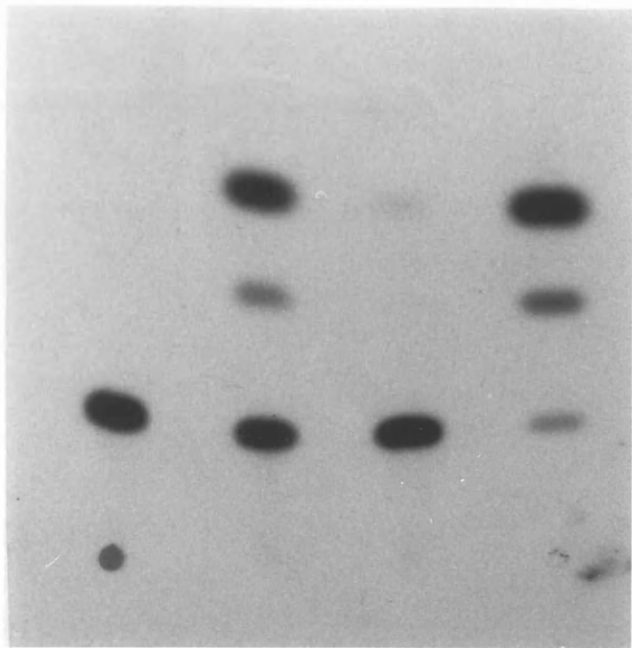




TABLE 6.10

Activation of the HSV tk promoter by Vmw 175 and Vmw 110

| Transfected DNA      | % acetylation<br>of [ $^{14}\text{C}$ ]-CM | $\mu\text{g}$ protein/<br>assay | Acetylated<br>[ $^{14}\text{C}$ ]-CM<br>n moles/mg/h |
|----------------------|--|---------------------------------|--|
| tk-CAT + pAT         | 0  | 25                              | 0  |
| tk-CAT + p175        | 46   | 25                              | 151  |
| tk-CAT + p111        | 1  | 25                              | 3  |
| tk-CAT + p175 + p111 | 94   | 25                              | 308  |

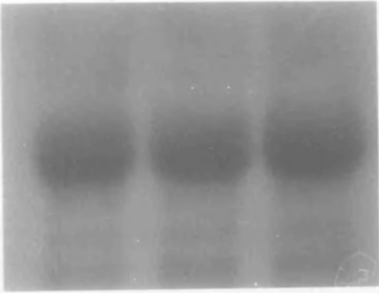
MCF-7 cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment and throughout the course of transfection. Cells were cotransfected with 10  $\mu\text{g}$  ptk-CAT together with 10  $\mu\text{g}$  of pAT or p175 or p111 or with 5  $\mu\text{g}$  p175 + 5  $\mu\text{g}$  p111. Plasmids are described in section 6.7.2. The methods for transfection, harvesting of cells, protein estimation and CAT assay are described in sections 2.2.15 - 2.2.17. Results are expressed as n moles acetylated [ $^{14}\text{C}$ ]-chloramphenicol (CM)/mg protein /h.

FIG. 6.16    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
TRANSFECTED WITH pAT (CONTROL) OR p175 or p111, PROBED  
FOR ER mRNA AND ACTIN mRNA.

(A) and (B) are two separate experiments.

The experiments were carried out as described in the legend to table 6.11.

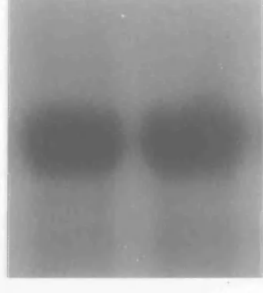
pAT p175 p111



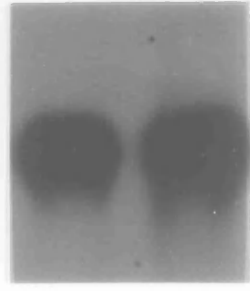
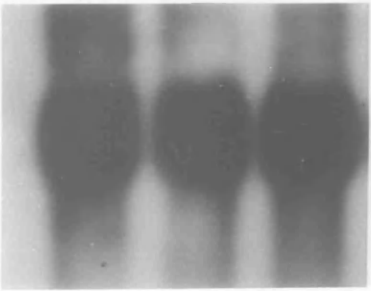
pAT



p175 p111



6 kb



2 kb

A

B

TABLE 6.11

Effect of Vmw 175 and Vmw 110 on ER mRNA levels

A.

| Transfected DNA | ER<br>(Area) | Actin<br>(Area) | <u>ER</u><br>Actin | ER mRNA as<br>% of Control |
|-----------------|--------------|-----------------|--------------------|----------------------------|
| pAT alone       | 4025         | 6967            | 0.58               | 100                        |
| pAT + p175      | 3840         | 5374            | 0.72               | 124                        |
| pAT + p111      | 4196         | 7255            | 0.58               | 100                        |

B.

| Transfected DNA | ER<br>(Area) | Actin<br>(Area) | <u>ER</u><br>Actin | ER mRNA as<br>% of Control |
|-----------------|--------------|-----------------|--------------------|----------------------------|
| pAT alone       | 2410         | 4592            | 0.52               | 100                        |
| pAT + p175      | 2981         | 4363            | 0.68               | 131                        |
| pAT + p111      | 3288         | 5085            | 0.65               | 125                        |

MCF-7 cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment and throughout the course of transfection. Cells were transfected (as described in section 2.2.15) with 20 µg pAT (control) or 10 µg pAT + 10 µg p175 or with 10 µg pAT + 10 µg p111. Plasmids are described in section 6.7.2. Cytoplasmic RNA was extracted, analysed and the results calculated as described in the legend to table 6.6. The results of two separate experiments are shown in tables 6.11 A. and B.

#### 6.7.4

#### Results

The results of cotransfecting the IE gene plasmids with tk-CAT are shown in fig. 6.15 and table 6.10. The control plasmid, pAT did not activate the tk-promoter. Plasmid p111 induced a very low level of CAT activity. Plasmid p175 caused a significant activation of the tk-promoter. The combination of p175 and p111 together with tk-CAT resulted in a greater stimulation of CAT activity than with either p175 or p111 alone.

The results of two experiments on the effect of Vmw 175 or Vmw 110 are shown in fig. 6.16 and table 6.11.

Vmw 175 caused a small (20 - 30%) increase in ER mRNA levels. The effect of Vmw 110 was variable as no stimulation of ER mRNA was seen in experiment (A) but a rise of 20% was seen in experiment (B). In a single experiment, the combination of p175 and p111 did not stimulate the level of ER mRNA (data not shown).

#### 6.7.5

#### Conclusions

1. CAT expression from the tk-promoter is greatly enhanced in the presence of Vmw 175 and activated at a low level by Vmw 110.
2. The combination of Vmw 175 and Vmw 110 together results in a greater stimulation of CAT expression from the tk-promoter than with either Vmw 175 or Vmw 110 alone.
3. Vmw 175 stimulates the level of ER mRNA only by a small amount (20 - 30%), the significance of which is unknown.
4. The effect of Vmw 110 on the level of ER mRNA is variable in these experiments.
5. The results of a limited experiment do not show a synergistic effect of Vmw 175 and Vmw 110 in activating the level of ER mRNA.

It should be noted that the above conclusions are based on a limited number of experiments. Efficiency of transfection is known to vary among experiments (Dr. R. Everett, personal communication; Dr. J. Macnab, personal communication; personal experience). Therefore, to make firm conclusions, several experiments showing consistent results are necessary. Due to the shortage of time, only a limited number of experiments were carried out but these are consistent with the interpretation that Vmw 175 and Vmw 110 do not account for the 100 - 200% increase in ER mRNA observed in HSV-1 infected cells.

The CAT results in MCF-7 cells (fig. 6.15, table 6.10) are similar to those described for the effect of Vmw 175 or Vmw 110 on the HSV gD or tk early promoters in HeLa cells (Everett, 1984, 1987; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). These authors came to the conclusion that Vmw 110 and Vmw 175 were able to independently activate HSV early promoters but that the greatest stimulation occurred when both Vmw 175 and Vmw 110 were present together.

The results described here indicate that p111 can activate the tk-promoter to a small extent while p175 gives a large activation of promoter activity. The level of expression seen in the presence of Vmw 175 alone is increased 2-fold when p111 is also present. Everett (1987) reported a 3- to 5- fold stimulation of CAT expression under the control of the HSV gD promoter in HeLa cells transfected with p175 or p111. The combination of p175 and p111 together gave a 20-fold greater stimulation than with either p111 or p175 alone. Thus the level of expression of the CAT gene is stimulated to varying degrees according to the type of promoter and/or the cell type used.

The level of stimulation of ER mRNA by Vmw 175 is not sufficient to account for the increase in ER mRNA induced by HSV-1 infection (section 6.6.2.1) although it is of a similar level to that found in

response to HSV-2 infection (section 6.6.2.2). Whether or not a stimulation of 20 - 30% is significant is unknown.

It is known that virus infection is a more efficient way of introducing viral DNA into cells than transfection. Deletion mutants for HSV-1 Vmw 175 (DeLuca et al., 1985), HSV-2 Vmw 175 (Smith and Schaffer, 1987a) and HSV-1 Vmw 110 (Stow and Stow, 1986; Sacks and Schaffer, 1987) could be used (with wild-type virus as control) to investigate the effect of HSV IE proteins on the levels of ER mRNA.

Clearly the results of the CAT assays (fig. 6.15 and table 6.10) and the RNA results (fig. 6.16 and table 6.11) are not comparable. It may be that the 5'-flanking sequences of the ER gene do not contain target sequences for HSV IE proteins. A target sequence for the binding of Vmw 175 in the HSV gD promoter has been identified (Faber and Wilcox, 1986). However, it is not known if this sequence is present in the regulatory sequences of the ER gene.

A more direct way of looking at the effect of IE proteins on expression of the ER gene would be to cotransfect p111 and/or p175 together with a plasmid containing the 5'-flanking sequences of the ER gene linked upstream of the CAT gene into ER negative cells. By this procedure, CAT expression under the control of the ER promoter could be easily measured.

## 6.8 EFFECT OF THE HSV VIRION COMPONENT, Vmw 65, ON THE EXPRESSION OF THE ER MESSAGE

### 6.8.1 Introduction

As previously described (section B1.6.3), the HSV virion component, Vmw 65 is a late protein which acts as a trans-inducing factor of HSV IE gene expression. Vmw 65 can also activate expression of heterologous genes in the presence of its target sequence, TAATGARAT (section B1.6.3). Since ER mRNA levels in MCF-7 cells are stimulated by infection with

HSV-1, HSV-2 and tsK (section 6.6), the possibility that Vmw 65 was involved in this stimulation was investigated.

### 6.8.2      Plasmids used in Transfection Studies

The following plasmids were kindly provided by Dr. C. Preston, Institute of Virology and are represented diagrammatically on the map of Vmw 65, fig. 1.5:

pMC1, pBamf      (Campbell et al., 1984) both encode wild-type Vmw 65

pBamfr:25 (pMC3) (Campbell et al., 1984) has the normal reading frame of the 1.9 kb mRNA for Vmw 65 disrupted by an 8 bp EcoR1 linker insertion which inactivates the trans-inducing function in transfection studies.

pMC7      was constructed by Dr. C. Preston (unpublished results) and contains an 8 bp insertion at the second Sal I site, which inactivates the trans-inducing function in transfection studies.

pMC1:ln8      was constructed by C. Ace (unpublished results) and contains an in-frame 4-amino acid insertion sequence at position 173 of the Vmw 65 amino acid coding sequence determined by Dalrymple et al. (1985). This insertion inactivates the trans-inducing function in transfection studies.

The plasmids pLW4 and pAT have already been described (sections 4.2.1 and 6.7.2 respectively). The plasmid pAT was used as carrier DNA to make up the total amount of transfected DNA to 20 µg.

### 6.8.3      Method

Cells maintained in DMEM + 2% HIDCC-FCS were transfected with each of



the above plasmids encoding wild-type or mutant forms of Vmw 65 and harvested 48h later. The relative level of ER mRNA in each case was compared by Northern blot analysis. As a positive control that Vmw 65 was able to activate gene expression in the presence of its target sequence, the above Vmw 65 plasmids were also cotransfected together with pLW4 and the level of CAT activity measured 48h later.

#### 6.8.4      Results

The results of two cotransfection experiments are shown in figs. 6.17A, 6.17B and tables 6.12A, 6.12B. Cotransfection of pLW4 and pMC1 resulted in an 8- or 9- fold stimulation of CAT activity. Cotransfection of pLW4 and pBamf resulted in a 12-fold stimulation of CAT activity. Thus Vmw 65 encoded by either pMC1 or by pBamf was able to activate the TAATGARAT element present in pLW4. The ability of the mutated forms of Vmw 65, encoded by pMC7, pMC1:ln8 and pBamfr:25, to stimulate CAT expression via the TAATGARAT element was greatly reduced compared with the wild-type level of expression, giving a 2- to 4- fold increase in CAT activity.

Transfection results with Vmw 65 or mutated forms of Vmw 65 on ER mRNA levels are shown in figs. 6.18A, 6.18B and tables 6.13A, 6.13B. All of the plasmids tested resulted in 100 - 200 % increase in ER mRNA except for pBamf. However, in a repeat experiment with a fresh preparation of pBamf obtained from Dr. M. Campbell, there was a 120% increase in ER mRNA level (data not shown).

#### 6.8.5      Conclusions

1. Plasmid encoded Vmw 65 stimulates CAT expression from pLW4 by 8- to 12- fold.
2. Mutated forms of Vmw 65 stimulate CAT expression from pLW4 to a reduced extent of 2- to 4-fold.

FIG. 6.17    (A) and (B)

CAT ASSAYS OF CYTOSOL EXTRACTS FROM MCF-7 CELLS COTRANSFECTED WITH  
PLASMIDS ENCODING WILD-TYPE OR MUTANT V<sub>mw</sub> 65 TOGETHER WITH pLW4.

Figures (A) and (B) are the results of two separate experiments.

The experiments were carried out as described in the legend to  
table 6.12.

Fig. 6.17 (A) is shown opposite.

Fig. 6.17 (B) is shown on the next page.

← p L W - 4 →

pAT

pMC1

pMC1  
ln:8

pBam  
f

pBam  
fr:25

3-ac

1-ac

CAP

ori

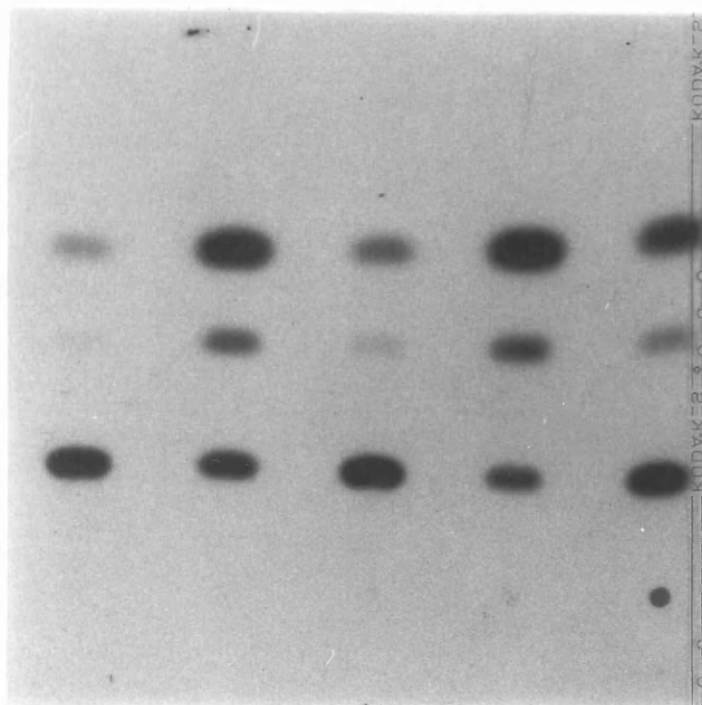


FIG. 6.17 (B)

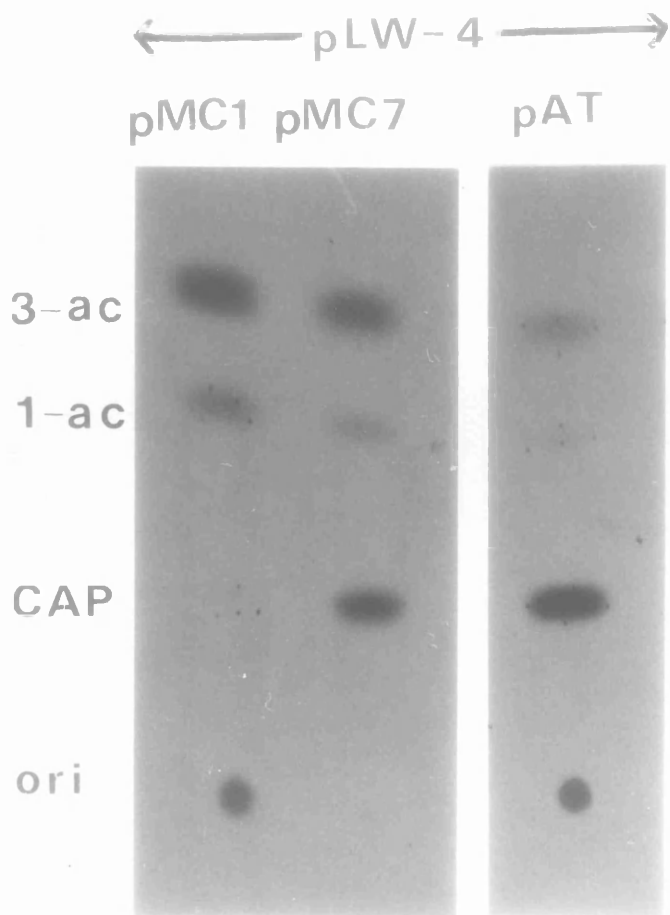


TABLE 6.12

Effect of Vmw 65 or mutant Vmw 65 on the activation of TAATGARAT

MCF-7 cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment and throughout the course of transfection. Plasmids used for transfection are described in section 6.8.2. Cells were transfected with (A) 10 µg pLW4 + 10 µg (pAT or pMC1 or pMC7) and (B) 10 µg pLW4 + 10 µg (pAT or pMC1 or pMC1:ln8 or pBamf or pBamfr:25). The methods for transfection, harvesting of cells, protein estimation and CAT assay are described in sections 2.2.15 - 2.2.17.

Results are expressed as n moles acetylated [<sup>14</sup>C]-chloramphenicol (CM)/mg protein/h and divided by the control (pLW4 + pAT) value for comparative results.

TABLE 6.12

Effect of Vmw 65 or mutant Vmw 65 on the activation of TAATGARAT

A.

| Transfected DNA | % acetylation of [ $^{14}\text{C}$ ]-CM | $\mu\text{g}$ protein/assay | Acetylated [ $^{14}\text{C}$ ]-CM n moles/mg/h | Fold Difference wrt control |
|-----------------|---|-----------------------------|--|-----------------------------|
| pLW4 + pAT      | 15                                      | 89                          | 14   | 1                           |
| pLW4 + pMC1     | 99                                      | 73                          | 111  | 8                           |
| pLW4 + pMC7     | 59                                      | 114                         | 42   | 3                           |

B.

| Transfected DNA | % acetylation of [ $^{14}\text{C}$ ]-CM | $\mu\text{g}$ protein/assay | Acetylated [ $^{14}\text{C}$ ]-CM n moles/mg/h | Fold Difference wrt control |
|-----------------|---|-----------------------------|--|-----------------------------|
| pLW4 + pAT      | 7.4                                     | 50                          | 12   | 1                           |
| pLW4 + pMC1     | 66                                      | 50                          | 108  | 9                           |
| pLW4+pMC1:ln8   | 12                                      | 50                          | 20   | 2                           |
| pLW4 + pBamf    | 88                                      | 50                          | 144  | 12                          |
| pLW4+pBamfr25   | 31                                      | 50                          | 50   | 4                           |

FIG. 6.18    (A) and (B)

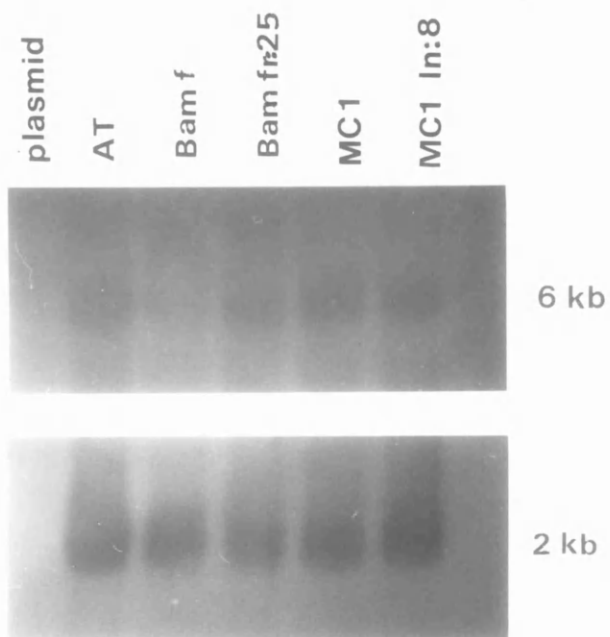
NORTHERN BLOTS OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS TRANSFECTED  
WITH PLASMIDS ENCODING WILD-TYPE OR MUTANT V<sub>mw</sub> 65, PROBED FOR  
ER mRNA AND ACTIN mRNA

Figures (A) and (B) are the results of two separate experiments.

The experiments were carried out as described in the legend to table 6.13.



**A**



**B**

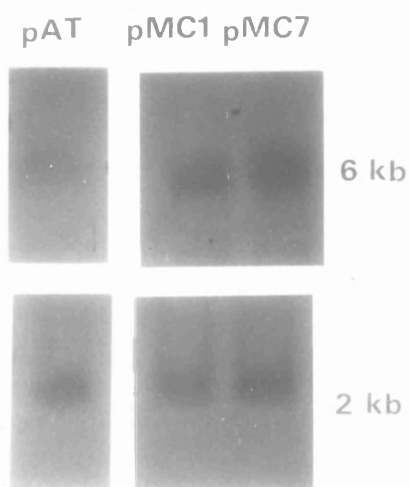


TABLE 6.13

Effect of Vmw 65 or mutant Vmw 65 on ER mRNA levels

A.

| Transfected DNA | ER<br>(Area) | Actin<br>(Area) | $\frac{\text{ER}}{\text{Actin}}$ | ER mRNA as<br>% of Control |
|-----------------|--------------|-----------------|----------------------------------|----------------------------|
| pAT alone       | 1485         | 3039            | 1.00                             | 100                        |
| pAT + pMC1      | 2553         | 2381            | 2.19                             | 219                        |
| pAT + pMC7      | 3309         | 2575            | 2.63                             | 263                        |

B.

| Transfected DNA  | ER<br>(Area) | Actin<br>(Area) | $\frac{\text{ER}}{\text{Actin}}$ | ER mRNA as<br>% of control |
|------------------|--------------|-----------------|----------------------------------|----------------------------|
| pAT alone        | 4011         | 7768            | 0.52                             | 100                        |
| pAT + pBamf      | 1524         | 3370            | 0.45                             | 87                         |
| pAT + pBamfr:25  | 2675         | 2439            | 1.10                             | 213                        |
| pAT + pMC1       | 3821         | 2740            | 1.40                             | 270                        |
| pAT + pMC1: 1n 8 | 3341         | 3461            | 0.97                             | 187                        |

MCF-7 cells were grown and transfected with plasmid DNA exactly as described in the legend to table 6.12. Cytoplasmic RNA was extracted, analysed and the results calculated as described in the legend to table 6.6.

3. Plasmids encoding either wild-type Vmw 65 or any mutated form of Vmw 65 all stimulate the level of ER mRNA by 100 - 200 %.

#### 6.8.6 Discussion

In agreement with the results of Campbell et al. (1984), Vmw 65 acts as a trans-inducing protein in MCF-7 cells in the presence of the TAATGARAT element. This ability is greatly reduced when mutations are introduced into the protein.

Both wild-type and mutated forms of Vmw 65 have the ability to stimulate the ER message. This result appears to contradict the result of the CAT assay. Several explanations are possible. It is possible that Vmw 65 is recognizing a sequence different from TAATGARAT, upstream of the ER gene resulting in activation of transcription of the ER gene and that the particular mutant proteins tested do not affect this function. Vmw 65 may be involved in stabilizing the ER message, a function which may not be affected by the particular mutations investigated here. Whatever the reason, Vmw 65 is acting differently in stimulating CAT expression from pLW4 than in stimulating the level of ER mRNA in the cells. These functions may reside on different domains of the protein.

Although the mutant proteins studied here showed a reduced ability to activate CAT expression, this ability was not completely abolished. Either the function involved in activating the TAATGARAT element was not totally inactivated by these mutations or some other domain of Vmw 65 has some trans-activating activity. This residual trans-activating activity may be activating the ER gene.

The level of stimulation of ER mRNA observed with Vmw 65 is comparable to that seen with HSV-1 infection. Moreover, as discussed in section 6.6.4.2, the cycloheximide results suggest that viral and cellular proteins are involved in increasing the level of ER mRNA. As Vmw 65 requires cellular proteins for its mechanism of action (Preston et al.),

inpress), it is possible that cycloheximide inhibits the HSV-1 activation of ER mRNA levels by inhibiting synthesis of Vmw 65 and the cellular protein(s) with which it interacts.

## 6.9            EFFECT OF CHEMICAL AND HEAT INDUCED STRESS ON THE EXPRESSION OF THE ER MESSAGE

### 6.9.1        Introduction

HSV infection results in an elevation of the levels of certain cellular proteins, notably the heat shock proteins (Notarianni and Preston, 1982; La Thangue et al., 1984), which in the latter case occurs at the level of transcription (Patel et al., 1986), (see also section B1.7.2).

An experiment was carried out to determine if the virus induced elevation of ER mRNA levels was related to the heat shock response at the level of transcription.

### 6.9.2        Method

Cells were maintained in DMEM + 2% HIDCC-FCS for 48h prior to the start of the experiment. The cells were then given the following treatments:

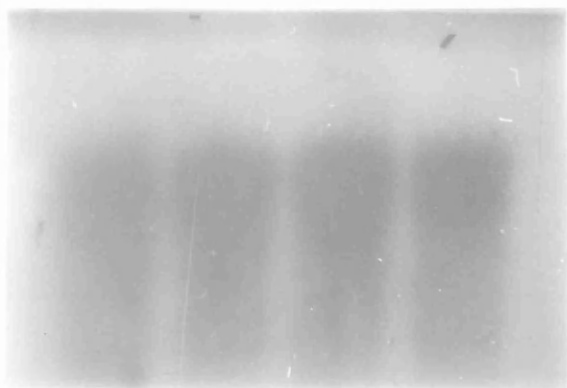
|                            |   |
|----------------------------|---|
| Control                    | Given fresh medium and harvested 4h later.  |
| Sodium arsenate<br>(NaArs) | Given fresh medium supplemented by 0.1 mM NaArs and<br>harvested 4h later.                                |
| Disulfiram<br>(DS)         | Given fresh medium supplemented by 0.3 $\mu$ M DS and<br>harvested 4h later.                              |
| Heat shock<br>(HS)         | Given fresh medium and incubated for 4h at 44°C then<br>transferred to 37°C for 30 min before harvesting. |

These conditions have been shown by Dr. J. Macnab to result in the expression of heat shock proteins (personal communication).

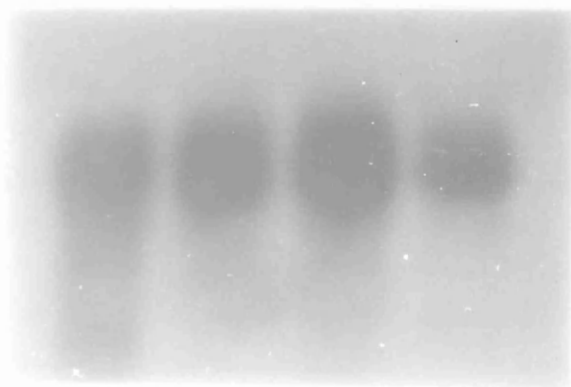
FIG. 6.19    NORTHERN BLOT OF TOTAL CYTOPLASMIC RNA FROM MCF-7 CELLS  
SUBJECTED TO HEAT OR CHEMICAL STRESS, PROBED FOR  
ER mRNA AND ACTIN mRNA

The experiment was carried out as described in the legend to table 6.14.

C DS Na HS  
Ars



6 kb



2 kb

TABLE 6.14

Effect of chemical and heat induced stress on ER mRNA levels

| Treatment | ER<br>(Area) | Actin<br>(Area) | $\frac{\text{ER}}{\text{Actin}}$ | ER mRNA as<br>% of Control |
|-----------|--------------|-----------------|----------------------------------|----------------------------|
| Control   | 7400         | 5111            | 1.45                             | 100                        |
| NaArs     | 7299         | 4800            | 1.52                             | 100                        |
| DS        | 8502         | 5905            | 1.44                             | 100                        |
| HS        | 5609         | 2151            | 2.61                             | 180                        |

MCF-7 cells were grown in 80 cm<sup>2</sup> flasks in DMEM + 10% FCS, then maintained in DMEM + 2% HIDCC-FCS for 2 days prior to the start of the experiment and throughout the course of the experiment. Cells were maintained as controls, treated with 0.1 mM sodium arsenate (NaArs) for 4h, treated with 0.3  $\mu$ M disulfiram (DS) for 4h or incubated at 44°C for 4h, then at 37°C for 30 min. Cytoplasmic RNA was extracted, analysed and the results calculated as described in the legend to table 6.6.

### 6.9.3

### Results

The results are shown in fig. 6.19 and table 6.14.

Treatment of cells with NaArs or disulfiram resulted in no change in the level of ER mRNA compared to control cells. In this particular experiment, heat shock treatment resulted in an 80% stimulation of the ER message. However, this was not a repeatable result and may have reflected the physiological state of the cells.

### 6.9.4

### Conclusion

General stress, as applied in these experiments, does not change the level of expression of the ER message.

## 6.10

### OVERALL CONCLUSIONS ON THE EFFECT OF HSV INFECTION ON ER mRNA LEVELS

Infection of MCF-7 cells by HSV-1 or by the mutant, tsK, at the non-permissive temperature, results in a 200 - 300 % increase in ER mRNA levels. Infection by HSV-2 results in a smaller increase (30%) in ER mRNA levels. The accumulation of ER mRNA is gradual, starting within 2h of infection and reaching maximum levels by 6 - 8 h post-infection. Treatment with  $10^{-8}$ M  $E_2$  for 24h results in a 50% rise in ER mRNA levels. With all three viruses, infection in the presence of  $10^{-8}$ M  $E_2$  results in a higher level of ER mRNA than with either virus or  $E_2$  alone. Cycloheximide results suggest the involvement of viral and cellular proteins. Transfection studies are consistent with the idea that Vmw 65 is involved in the virus mechanism of action. The stimulation of ER mRNA levels is not linked to the stress response (with the exception of a single experiment which showed an increase in ER message in response to elevated temperature).



## GENERAL CONCLUSIONS AND DISCUSSION

The aim of this thesis was to investigate the possible interaction of steroid hormones and HSV. The studies were based on the association of HSV with cervical cancer (section C1.3), the role of hormones in the normal development and pathology of the cervix (section C1.2) and the increased risk of developing CIN in women using the contraceptive pill (section C1.1.3). Four aspects of steroid hormone /HSV interaction were studied:

- (a) The effect of hormones on virus growth (section 3)
- (b) The effect of hormones on virus gene expression (section 4)
- (c) The effect of virus infection on steroid receptor levels (section 5)
- (d) The effect of virus infection on steroid mRNA levels (section 6)

The results are discussed in detail at the end of each set of experiments. The most important points will be emphasized in this final discussion.

The object of the experiments on the growth of HSV-2 in primary cells (section 3) was to mimic the in vivo situation as closely as possible, therefore primary cells, physiological concentrations of hormones and low multiplicity of infection were used. Neither oestrogen nor progesterone significantly affected HSV-2 titres in either primary cervical cells or in ZR-75-1 cells. As discussed in section 3.6, stromal cells may influence the response of epithelial cells to hormones or virus infection in vivo, possibly by the secretion of growth factors (Cuhna et al., 1987) as has been shown for breast cells Schor et al., 1987). The effect of hormones and/or virus infection on stromal cells was not investigated.

The results of section 4 show that in transient, transfection assays, in MCF-7 or ZR-75-1 cells, neither oestrogen nor progesterone modulate expression of the CAT gene under the control of an HSV promoter and upstream activator sequence, TAATGARAT (which shows partial homology

to the consensus binding site for chicken oviduct PR), even although both MCF-7 and ZR-75-1 cells contain cellular factors which specifically complex with TAATGARAT DNA in vitro. These results are discussed in detail in section 4.4.4 and 4.5.6. Points to be noted are that (a) both the TAATGARAT sequence and chicken PR binding sequence are consensus sequences and only show partial homology with each other; (b) it is known, at least in the case of the TAATGARAT sequence (Bzik and Preston, 1986), that flanking sequences are important for full activation of transcription via the TAATGARAT element; (c) chicken and human cells contain different cellular factors which could affect binding of PR to its target sequence. Therefore, it is probable that the precise DNA core and flanking sequences, chromatin configuration and cellular factors involved in binding PR are specific and are not mimicked by the HSV IE activator sequence which is the target sequence for the virion trans-inducing factor, Vmw 65. As discussed in section A1.7.4, tissue specific transcription factors are important for regulation of steroid inducible genes in the chicken oviduct.

The other possible reasons (discussed in section 4.4.4) for the failure of hormones to activate gene expression in the presence of the TAATGARAT element include the possibility that the concentration of PR in the cells was too low or was not at a constant level or that transfection efficiency was not high enough. It would be worthwhile repeating these experiments by cotransfection of the pLW plasmids with a plasmid encoding the PR under the control of a strong, inducible promoter to ensure high, constant levels of PR synthesis.

In the context of these experiments, it is interesting to note that Gloss et al. (1987) found that the upstream regulatory region of HPV-16 genomic DNA contains a sequence element with a large degree of homology to known GRE's of glucocorticoid regulated genes, binds GR in vitro and is protected from DNase I cleavage and dimethylsulphate (DMS) methylation by GR. However, when HPV-16 RNA from CaSki cells (which contain and express endogenous HPV-16 genomes; Smotkin and Wettstein, 1986) treated with or without dexamethasone was quantitated

on Northern blots, no difference was seen between control and dexamethasone treated cells. Moreover, when a positive control plasmid containing a known GRE linked to the CAT gene was transfected into CaSki cells with or without dexamethasone, no increase in CAT expression was observed in the presence of dexamethasone. The authors concluded that this may be due to a low endogenous level of GR in CaSki cells. It could also be due to incorrect flanking sequences or the wrong DNA configuration.

Section 5 describes biochemical and immunological analysis of detectable ER and PR levels in ZR-75-1 and MCF-7 cells in response to E<sub>2</sub> stimulation and/or HSV infection. Both HSV and E<sub>2</sub> caused a fall in ER levels to about 10% of the control value. E<sub>2</sub> caused the levels of PR to rise, thus indicating that the cells were responding as expected to E<sub>2</sub>. HSV infection resulted in a reduction of PR levels, thus indicating that the mechanisms of action of E<sub>2</sub> and HSV are different. These results are discussed fully in sections 5.3.4 and 5.4.5. The reason for the drop in receptor levels may be due to modification of the receptor in a way which prevents ligand binding or recognition by the monoclonal antibody so that the receptor is no longer detectable by these means. The virus may bind the receptor so that it is no longer able to bind ligand and the epitope recognized by the antibody may be masked.

Attempts to in vitro translate and immunoprecipitate the ER protein from total cellular RNA were unsuccessful (section 5.5). As discussed in section 5.5.5, this may be due to the low abundance of the ER message or the fact that only one monoclonal antibody to the ER was used (which is known to recognize the nuclear bound form of the receptor but may not recognize the newly synthesized receptor). In contrast, Walter et al. (1985), using hybrid-selected ER mRNA and a combination of four monoclonal antibodies managed to in vitro translate and immunoprecipitate a protein of mol. wt. approximately 65 000.

Section 6 describes the effects of E<sub>2</sub> stimulation, HSV infection and transfection of HSV regulatory proteins on ER mRNA levels. Overall conclusions are given in <sup>n</sup>section 6.10. Results are discussed in detail in sections 6.4.4, 6.7.6 and 6.8.6. The ability of E<sub>2</sub> or HSV to increase the cellular levels of the ER message seems to contradict the results with the ER protein levels (section 5). However, if it is true that the reason for the fall in detectable ER levels is due to modification of the receptor so that it can no longer bind ligand and is no longer recognized by the antibody, then the problem lies in the assay methods used to detect the ER protein. Alternatively, (a) there may be a post-transcriptional block such that the extra ER mRNA available is not translated into protein or (b) it is not the complete ER message that is being detected by the probe in hybridization experiments. A few examples of increased message levels without a corresponding increase in protein levels do exist, including the trans-acting protein, tat, of the human immunodeficiency virus (HIV), (J. Nelson, personal communication). Expression of the tat message seems to alter expression of other genes in a way that is not yet understood. The tat message possibly binds to other messages resulting in stabilization of those messages.

The activation of a cellular message is an unusual consequence of HSV infection. Most macromolecular synthesis is turned off as a result of HSV infection (section B1.7.1). There are two examples of transcriptional activation of cellular genes by HSV. One is a heat shock protein (Patel et al., 1986) and the other is of unknown function (Kemp et al., 1986). Certain cellular polypeptides are upregulated by HSV infection, including the heat shock proteins (Notarianni and Preston, 1982; La Thangue et al., 1984) and some polypeptides of unknown function in HSV-2 infected and HSV-2 transformed rodent cells (Macnab et al., 1985). Most other cellular gene and protein expression is turned off.

As previously discussed (sections B1.7.1 and 6.5.1), there has been great variability in results of shutting off of cellular messages by

HSV, depending on the virus strain and cell type used. It is important to stress that the experiments described in section 6 were done in breast epithelial cells, but not in cervical epithelial cells which are infected with HSV in vivo. There was no evidence for the switching off of actin message by HSV-1 or HSV-2 but tsK was capable of significant depression of actin mRNA (section 6.6.2.3). As discussed in section 6.6.4.2, this effect of tsK may be due to the synthesis of an abnormal form of Vmw 175, also known to induce the heat shock response (Russell et al., 1987).

The effect of E<sub>2</sub> on the expression of the ER message showed a cyclical effect (section 6.4.2.1). This is probably linked to the cell cycle: The E<sub>2</sub> binding capacity of breast cancer cells varies with the cell cycle (Chua et al., 1986). It is known that genes which are switched on early in the cell cycle are expressed at a much lower level at later stages in the cell cycle and then more fully again after cell division (Wheatley, 1982).

The importance of cell type in testing the response of HSV IE-CAT constructs to viral trans-acting proteins (Vmw 175, Vmw 110 and Vmw 65) has been demonstrated by Gelman and Silverstein (1987b). They found that IE-CAT constructs showed a different basal level of transcription and differed in the level of their response to trans-acting viral proteins in Vero (African green monkey kidney cells) and HeLa cells, even although the virus grows equally well in both cell lines. Therefore, it is important to consider both viral and cellular factors in studying regulation of transcription by virus.

The ability of HSV to increase the level of the ER message may have important consequences. The ER plays a central role in the mechanism of action of oestrogen. The biological responses of cells to <sup>o2</sup>~~eo~~estrogen are described in sections A1.5 and A1.6. Overall, oestrogen promotes cell growth and division. The fact that HSV affects the level of the ER message may have implications in regulation of cell growth. In view of the association of HSV with CIN (section C1.3) and one of the

proposed mechanisms for transformation by HSV being activation of cellular genes (Macnab, 1987), it is very interesting to find that HSV increases the level of a cellular message of a protein directly involved in growth control. E<sub>2</sub> directly affects cell growth by changes in RNA, protein and DNA synthesis (section Al.5.4) and indirectly by influencing the secretion of growth factors (section Al.6).

As described in section Al.8, the amino acid sequences of steroid and thyroid hormone receptors, vitamin D<sub>3</sub> receptor and retinoic acid receptor all share high homology with the v-erb A oncogene of AEV, especially in the putative DNA - binding domain. The v-erb A oncogene is not sufficient by itself to transform erythroblasts but potentiates the action of the v-erb B oncogene (section Al.8.4). Recent experiments (Boucher et al., 1988), using oligopeptide antisera directed against specific domains of the v-erb A protein in immunofluorescence and immunoprecipitation studies on subfractionated cell extracts, have shown both cytoplasmic and nuclear localization of v-erb A. Both forms of the protein were able to bind DNA. A mutation within the v-erb A coding region which inhibited DNA binding and nuclear localization, also inhibited the ability of the v-erb A protein to potentiate erythroid transformation, consistent with the model of v-erb A as a transcriptional regulator. Moreover, the mutation which inhibits nuclear localization of v-erb A occurs within a similar sequence and in a position equivalent to the site of the nuclear localization signal in the GR (Picard and Yamamoto, 1987). Deletion of the hormone - binding domain of the GR leads to a constitutively active receptor which does not bind hormone (Godowski et al., 1987). The v-erb A protein has lost the ability to bind hormone, in contrast to its cellular counterpart, c-erb A which is a high affinity thyroid hormone receptor (section Al.8.4). Sap et al. (1986) have proposed that the v-erb A protein functions in oncogenesis as a constitutively "turned on" derivative of a hormone receptor and this view is supported by Boucher et al. (1988). Presumably, v-erb A acts by modulating transcription of specific target genes within the nucleus of the transformed erythroid cell.

In view of the homology between v-erb A and steroid and retinoic acid receptors (retinoic acid affects cell differentiation) and the results of this thesis showing elevation of the levels of the ER message by HSV infection, it would be interesting to test the ability of HSV to activate the expression of other normal cellular genes and cellular oncogenes.

The oncogene, v-ras<sup>H</sup>, when transfected into MCF-7 cells, is able to bypass the oestrogen dependence on growth of these cells as tumours in vivo (Kasid et al., 1985) probably because the cells can now secrete growth factors capable of supporting their own tumour growth in vivo (Kasid et al., 1987). Human breast tumours sometimes lose their hormone dependence and this may reflect a change to control by autonomous growth factors. Transfection of v-ras<sup>H</sup> into MCF-7 cells also affects their ability to interact with and cross basement membrane components (Albini et al., 1986). The ability of tumour cells to cross basement membranes is an important step in the progression to malignancy.

The mechanism of action of oestrogen in cell or tumour growth seems to be linked indirectly with the secretion of growth factors either by the oestrogen target cell or by a neighbouring cell (section A1.6). Thus it would be interesting to look at the effect of HSV on growth factor mRNA levels.

In conclusion, the studies described in this thesis show no effect of steroid hormones on HSV growth or gene expression. However, HSV infection has an effect on steroid receptor and message levels. The stimulation of ER mRNA levels by HSV appears to be mediated by the HSV trans-inducing factor, Vmw 65. Transcriptional activation of cellular genes is an important mechanism of action of certain oncogenes. In view of the association of HSV with cervical carcinoma, it is possible that HSV activates the expression of certain cellular genes involved in growth control, thus playing a role in the progression to abnormal cell growth.



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## 8. REFERENCES

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